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THE PHOTOSYNTHETIC CYCLE

Melvin Calvin

March 21, 1955

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ABSTRACT

A cyclic sequence of transformations, including the carboxylation of RuDP (ribulose diphosphate) and its re-formation, has been deduced as the route for the creation of reduced carbon compounds in photosynthetic organisms. With the demonstration of RuDP as substrate for the carboxylation in a cell-free system, each of the reactions has now been carried out independently in vitro. Further purification of this last enzyme system has confirmed the deduction that the carboxylation of RuDP leads directly to the two molecules of PGA (phosphoglyceric acid) involving an internal dismutation and suggesting the name "carboxydismutase" for the enzyme. As a consequence of this knowledge of each of the steps in the photosynthetic CO_2 reduction cycle, it is possible to define the reagent requirements to maintain it. The net requirement for the reduction of one molecule of CO_2 is four equivalents of [H] and three molecules of ATP (adenine triphosphate). These must ultimately be supplied by the photochemical reaction. Some possible ways in which this may be accomplished are discussed.

The requirement of four equivalents of [H] and three molecules of ATP for the reduction of each molecule of CO_2 in the photosynthetic carbon-reduction cycle suggests the possibility that respiration may contribute some of the energy required for photosynthesis by supplying some of the ATP. This possibility was studied by measuring the quantum requirement of photosynthesis at various ratios of photosynthesis rate to respiration rate. Both corrected and uncorrected quantum requirements approach an experimental value of 7.4 with increasing photosynthetic rates, while the corrected rate approaches 4 as the photosynthetic rate approaches zero. This may indicate a contribution of respiratory energy, probably as ATP, to photosynthesis.

We thus have the possibility of an interplay between the photosynthetic apparatus in the chloroplasts and the rest of the energy-converting apparatus of the cell outside it, at least at two points; namely, a number of intermediates in the carbon-reduction cycle that are common to both, as well as generalized energy-storage chemicals such as ATP, which are generated and used by both.

A study of the effect of light intensity on the appearance of radiocarbon in a number of pools directly associated with the tricarboxylic acid cycle has implicated thioctic acid in a step very closely related to the photochemical act. Direct experiments designed to determine just how close

(*) The work described in this paper was sponsored by the U.S. Atomic Energy Commission.

that association is have been performed. These involved the observation of increased quantum yield for photosynthesis and oxygen production by added thioctic acid under a variety of conditions. They have led to a new conception of the nature of the photochemical act as it occurs in the lamina of the subchloroplast structural units (grana). The suggestion is that the absorption of light creates within the chlorophyll-containing layer conduction electrons, one per quantum, which are separated from the remaining "holes" because of the structure of the lamina. The holes are trapped by donation of electrons from water molecules, or their close relatives, while the electrons are accepted by the sulfur atoms of a thioctic acid-like compound to produce a dithiol which, in turn, can pass its hydrogen on to other carriers, ultimately to PGA.

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INTRODUCTION

As you know, the problem of photosynthesis is the one of determining how the green plants store electromagnetic energy in a chemical form by performing this chemical transformation,

$$CO_2 + H_2O \xrightarrow{h\nu} (CH_2O)_x + O_2,$$

converting the carbon dioxide and water into carbohydrates and molecular oxygen. This reaction has been separated into two pretty well distinct parts, both theoretically and actually physically: first, the quantum conversion process involving the splitting of water into some sort of reducing agent plus one-half mole of oxygen;

$$H_2O \xrightarrow{h\nu} [H] + 1/2\ O_2,$$

followed by the reduction of carbon dioxide, using this reducing agent [H] to produce carbohydrates:

$$CO_2 + [H] \longrightarrow (CH_2O)_x.$$

Now what I have to say this evening is concerned mostly with this last half of the operation, of which we have been able to draw a complete map, using carbon-14 tracers. This is a work of a period of about eight or nine years and of a laboratory of fluctuating population and size.

(*) Most of the work described herein has been documented in (1) "The Path of Carbon in Photosynthesis. XXI." by J. A. Bassham, A. A. Benson, L. D. Kay, A. Z. Harris, A. T. Wilson, and M. Calvin, J. Am. Chem. Soc. 76, 1769 (1954) and (2) "Photosynthesis" by J. A. Bassham and M. Calvin, a chapter in Currents in Biochemical Research, edited by D. E. Green, to be published by Interscience Publishers, Inc. The new material given in this paper is the work of J. R. Quayle, R. C. Fuller, J. Mayoudon, K. Shibata, J. A. Bassham, and D. F. Bradley, and is particularized at the proper point.

(**) Presented as Edgar Fahs Smith Memorial Lecture, Philadelphia, Pennsylvania, March 17, 1955.

FIRST PRODUCTS

The essential character of the experiment is simple: to set up a plant in a steady state of photosynthesis, feed it carbon dioxide with labeled carbon in it, and trace the path of carbon as it goes through a sequence of transformations. The way in which this has been done has been described a number of times. So I am going to go through quite rapidly the sequence of operations that lead to the results at the end.

The steady state is set up by using green algae in a lollipop (Fig. 1) and the radiocarbon is fed in at a given time -- over a period of some seconds to minutes. Then the algae are killed by dropping them into hot alcohol and the alcohol extract is examined for radioactive compounds. This examination is performed by means of paper chromatography and radioautography, Fig. 2 shows the results of a 60-second experiment, and you see there are some twenty or more compounds formed from the radioactive carbon, and it is quite clear that 60 seconds is too long a time. We therefore shorten the time progressively; Figure 3 shows a 10-second experiment and by this time one compound is predominant. In fact, if the experiment is extrapolated back to zero time all the radioactive carbon turns out to be in the phosphoglyceric acid. Thus we have been able to identify the first compound into which carbon dioxide is incorporated by photosynthesis.

Figure 4 shows the distribution of the labeled carbon in the three carbon atoms of the glyceric acid obtained from the phosphoglyceric acid-- this happens to be a 15-second experiment. Half of the activity is in the carboxyl group and the other half is split equally between the other two carbon atoms. From the same experiment a sugar molecule was obtained, and it also was degraded, and the distribution of carbon in it was found to be very much the same as it is in the three-carbon pieee of glyceric acid. This immediately suggests that the six-carbon piece is made from the two three's by putting the two carboxyl groups together, which, of course, is simply a reversal of the well-known aldolase split of fructose diphosphate in the glycolytic sequence, a part of which is shown in Fig. 5. Here the glyceric acid is reduced with the hydrogen from the photochemical reaction to glyceraldehyde, which is then isomerized to the ketotriose, and the two of them hooked together to give hexose. Thus, the two carbon atoms which were originally carboxyl finally fall in the middle of the hexose chain. It's quite clear that there must be something that accepts the carbon dioxide to form the glyceric acid and, furthermore, that that something must be regenerated from the PGA (phosphoglyceric acid), triose phosphates, and hexose phosphates, or something else formed from them.

Sucrose Synthesis

In passing, it might be worth describing what we know about the fate of the fructose diphosphate and how it gets to the common table sugar, sucrose. This was worked out by identifying several of the additional spots on the paper and showing their relationship to fructose phosphate and to sucrose. Figure 6 shows that relatipnship as we found it. Here are shown the phosphoglyceric acid, the fructose diphosphate, and evidence of the various transformations which lead ultimately to glucose-1-phosphate. This reacts with uridine triphosphate to make uridine diphosphoglucose. This uridine diphosphoglucose (UDPG) is found on the paper, with glucose labeled very rapidly, and it then can react in either of two ways: either with fructose-1-phosphate to form sucrose phosphate (which was found on

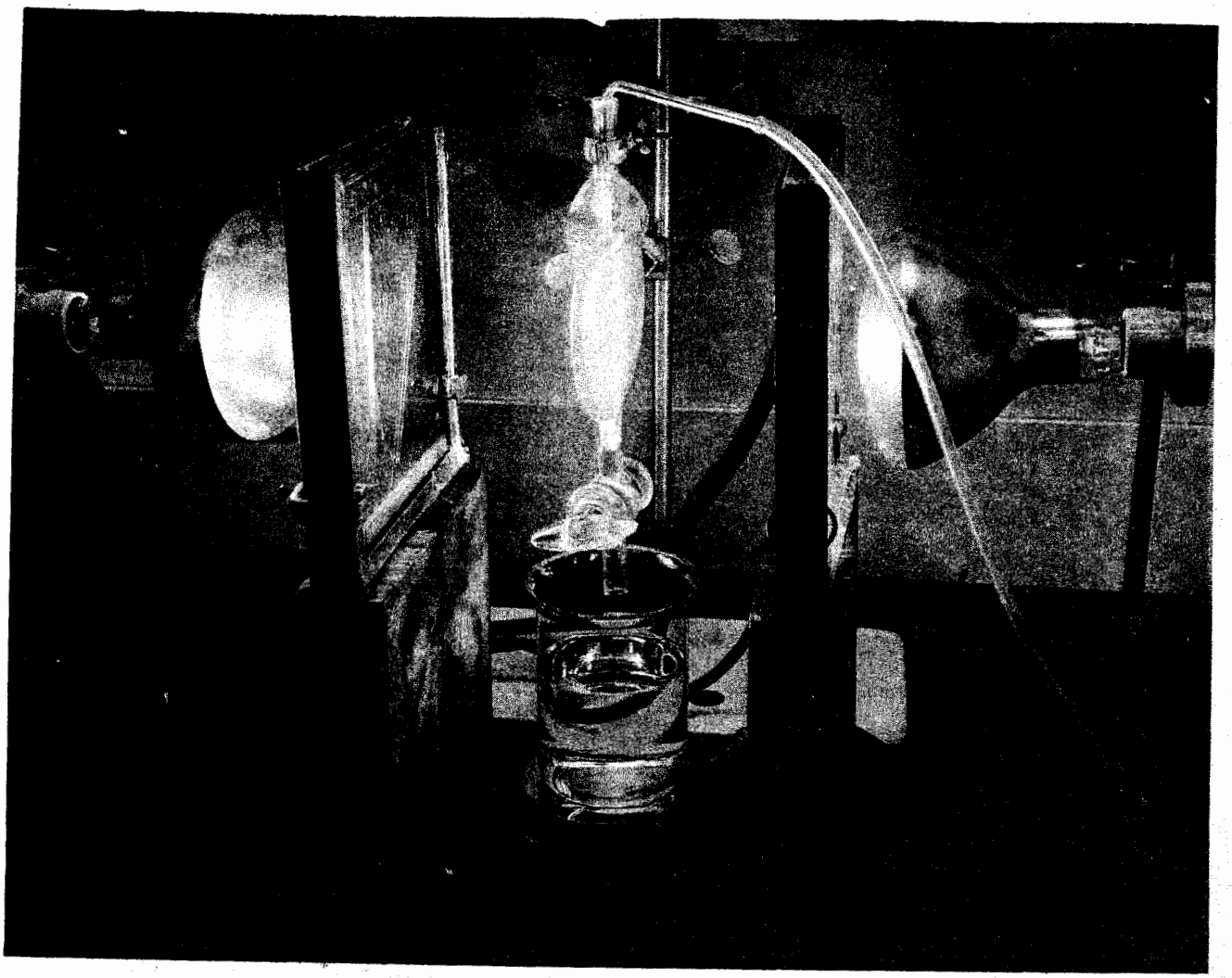

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Fig. 1 "Lollipop"

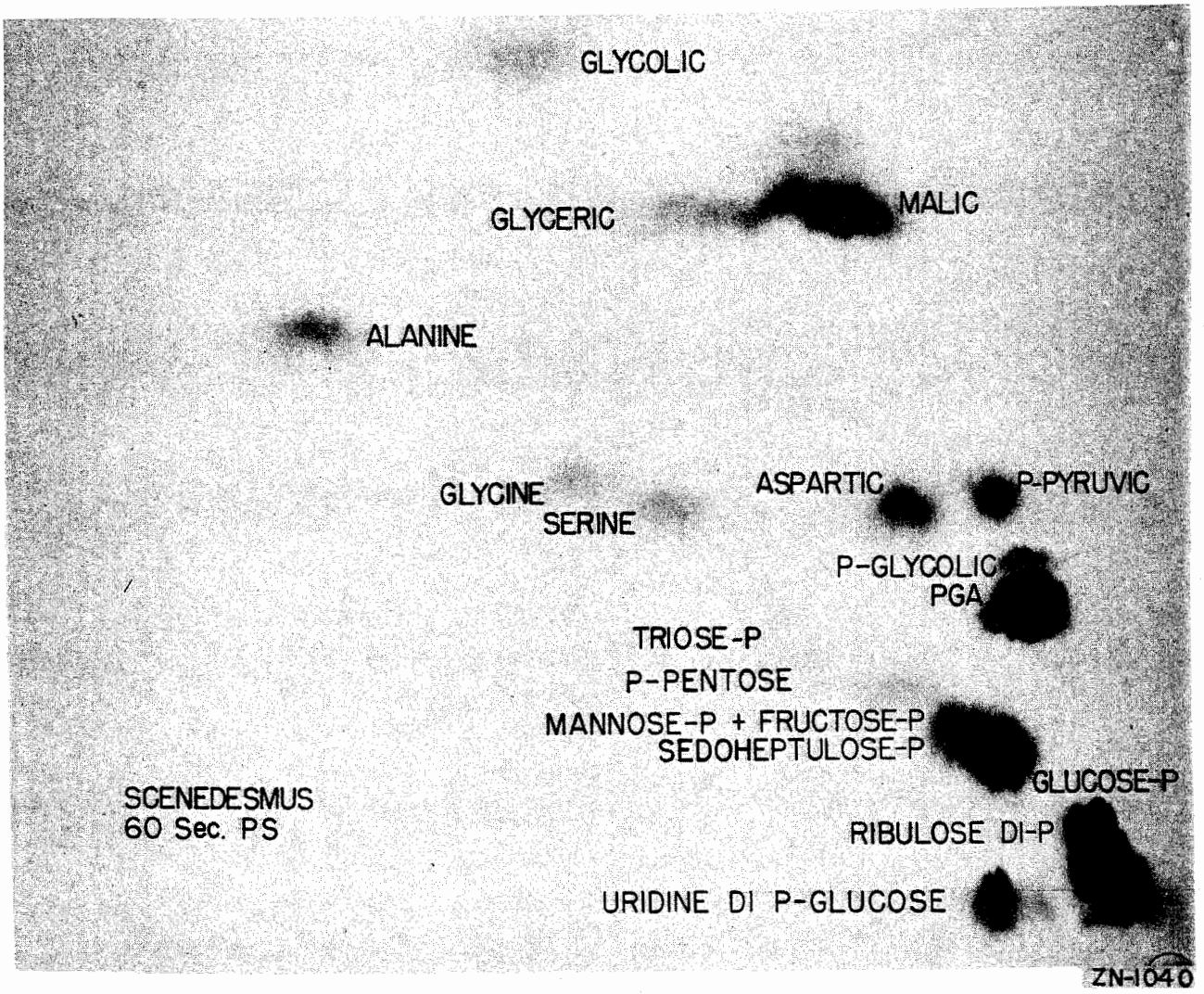

Fig. 2 Chromatogram of Extract From Algae Indicating Uptake of Radiocarbon During Photosynthesis (60 Seconds)

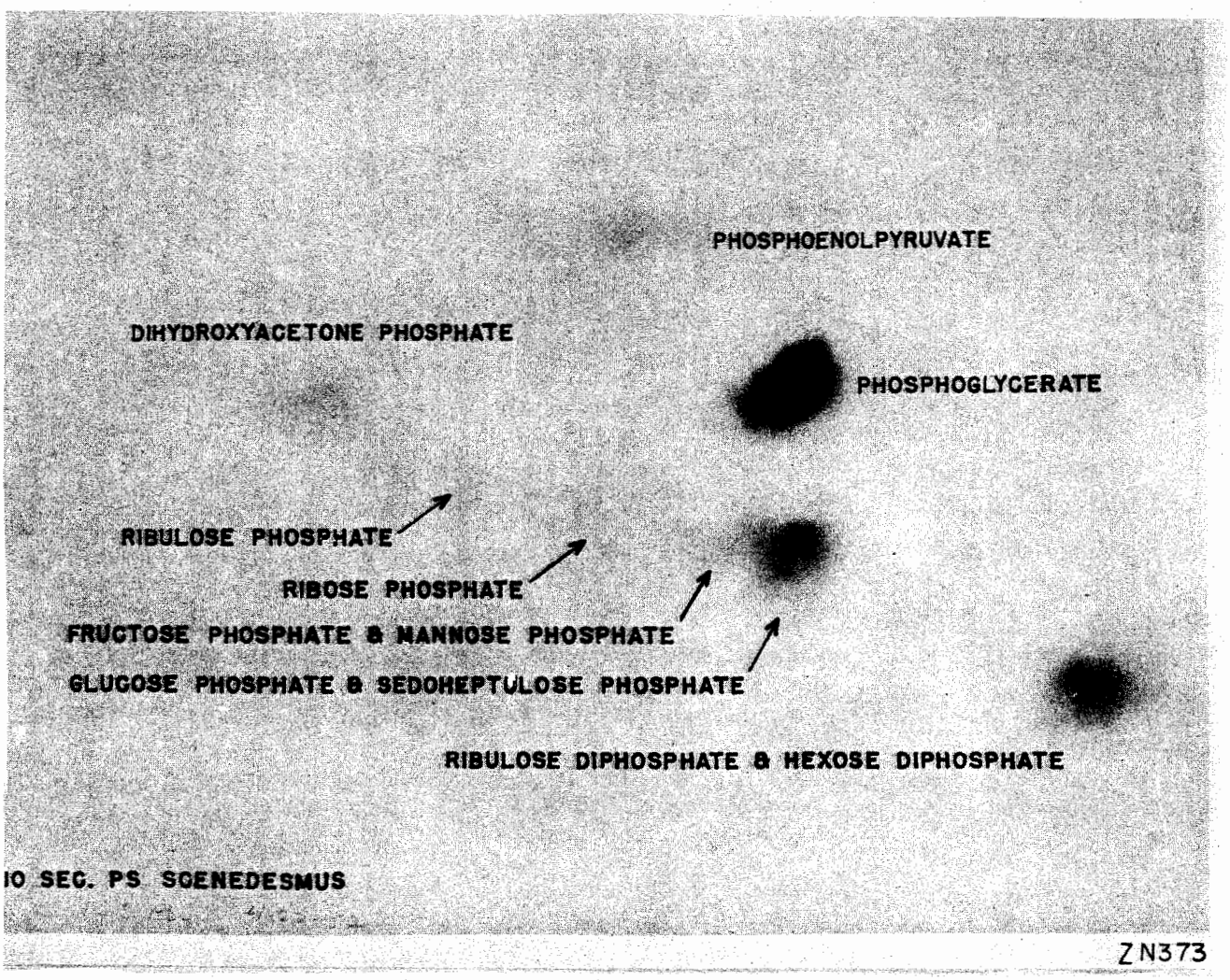

Fig. 3 Chromatogram of Extract From Algae, Indicating Uptake of Radiocarbon During Photosynthesis (10 Seconds)

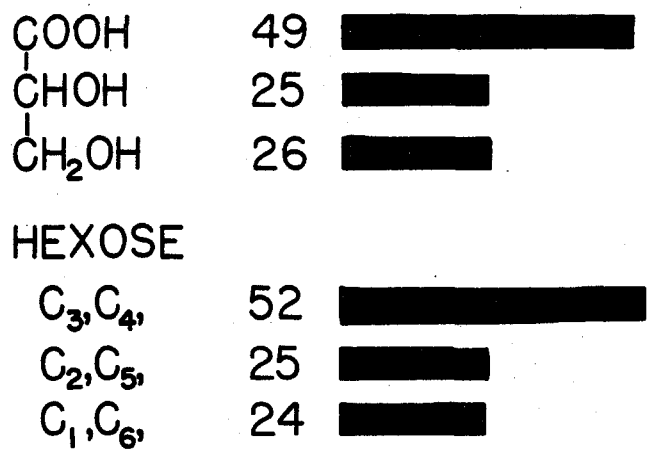

Fig. 4 Distribution of Labeled Carbon in Photosynthesis Experiments.

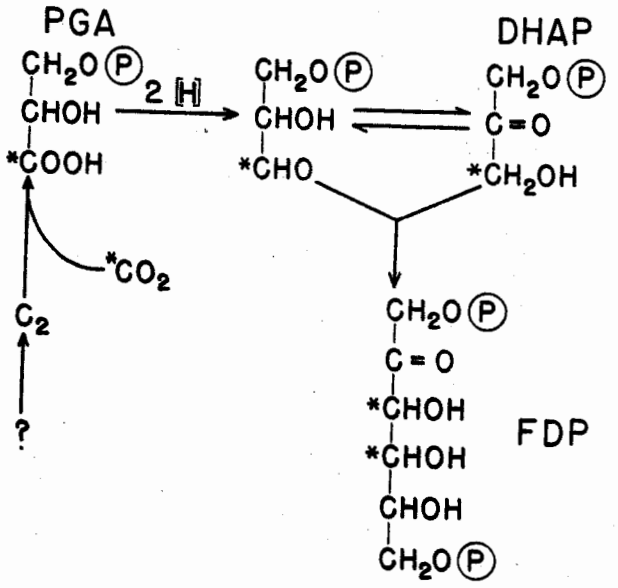

Fig. 5 Path of Carbon From CO_2 to Hexose During Photosynthesis

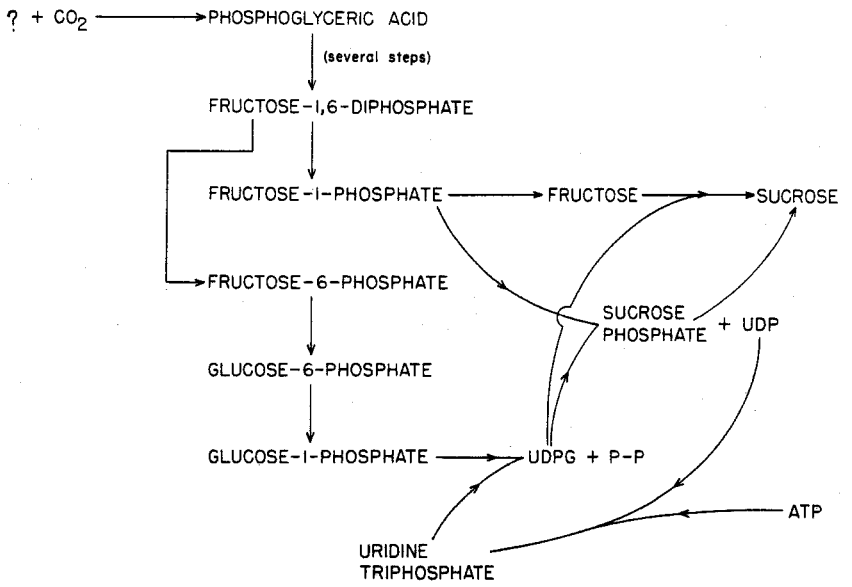

Fig. 6 Proposed Mechanism for Formation of Sucrose with Uridine Diphosphoglucose

the paper) which then is phosphatased to sucrose, or it can react directly with free fructose to form sucrose in one operation. However, since we never see any free labeled fructose, the first of these alternatives appears to be the major pathway for green leaves. And I might add that an enzyme performing the reaction

UDPG + fructose phosphate ——→ sucrose phosphate

has recently been prepared in a partially purified state by Leloir in Argentina. Figure 7 shows the structual formula for the UDPG, and its reaction with fructose-1-phosphate -- a sort of a double-decomposition reaction giving uridine diphosphate and sucrose phosphate with the phosphate on the Number 1 carbon atom of the fructose moiety. The phosphate is finally taken off to give sucrose. This appears to be the common route to sucrose; it is one of the major synthetic reactions in agriculture, and provides the substrate for a wide variety of other transformations.

C_5 and C_7 Sugars

Let us return to the problem of where and how the acceptor of the carbon dioxide is created, or regenerated, from these various other substances we have seen. In trying to pursue the original plan further -- that is, to see if we could outline the sequence of steps that lead to the regeneration by simply taking out the PGA and extrapolating back to zero time for the next compound which would appear at 100% and which should be the next one in the sequence -- we found it to be impossible. That is, three or four compounds extrapolated out, suggesting that not one was the next after PGA and triose, but several simultaneously formed from this one precursor -- this triose precursor. Indeed, the compounds which showed up on further analysis, in addition to the six-carbon sugar (which you have seen), were a five-carbon and a seven-carbon sugar - the three-, five-, six-, and seven-carbon sugars, then, were found.

A detailed analysis of the distribution of radioactivity among the carbons of these sugars is shown in Fig. 8. Here, besides PGA, are the five-carbon sugar, ribulose diphosphate (RuDP); the seven-carbon sugar, sedoheptulose phosphate (SMP); and the skeleton of a six-carbon sugar, corresponding either to glucose or fructose (these are the major six-carbon sugars that we find). The stars give some indication of the order of appearance of radioactive carbon in these compounds, and it was from an analysis of this sort that we were able to reduce relationships between the various compounds.

In much the same way as we deduced the relation between the three-carbon PGA and the six-carbon hexoses we were able to deduce the relationships between the five- and seven- and six- and three-carbon compounds that are shown here. Now actually it is quite clear at a glance that there is no simple structural relationship between the five- and the seven-carbon compounds and the other two -- nothing, at least, as simple as the relationship between the three-carbon PGA and the six-carbon hexose which appears when the two PGA's are simply joined together. There is no sequence in the C_5 or C_7 that could be considered as simply the intact C_3 or the intact C_6, respectively. It wasn't until we realized that the C_5 might have more than one origin that we were able to deduce a possible route for its formation. This is shown in Fig. 9. By taking two carbons off the top of the C_7 and adding

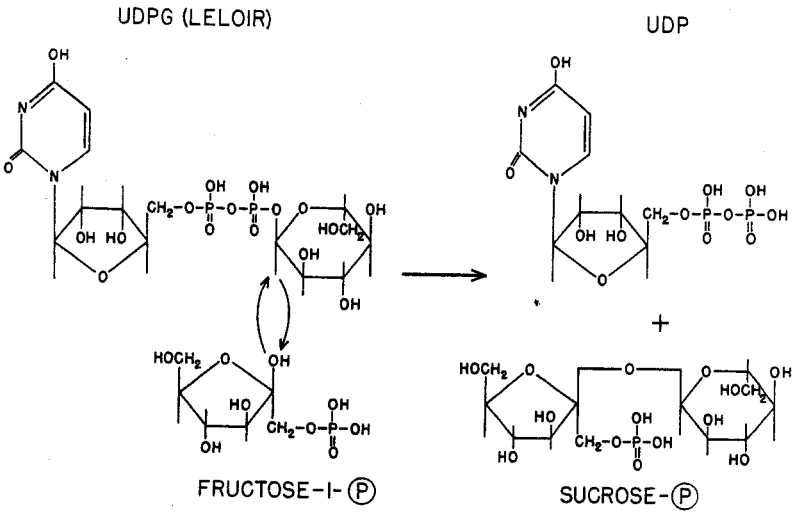

Fig. 7 Uridine Diphosphoglucose Reaction with Fructose Phosphate

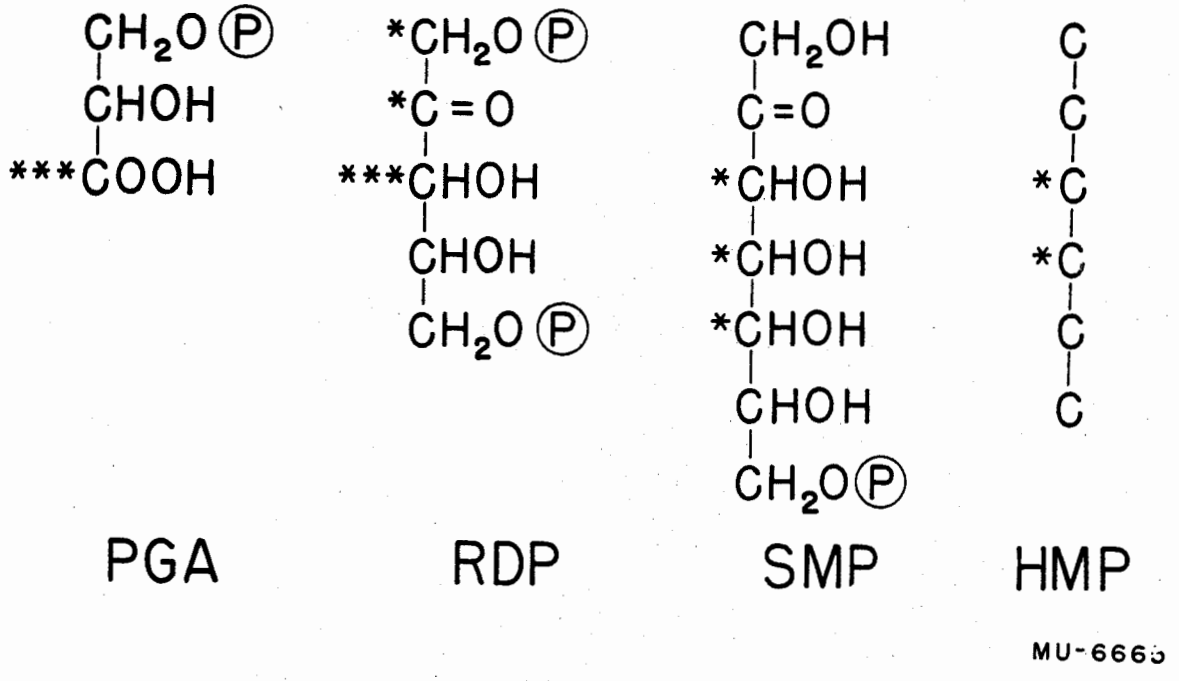

Fig. 8 Distribution of Radioactive Carbon in Certain Sugars

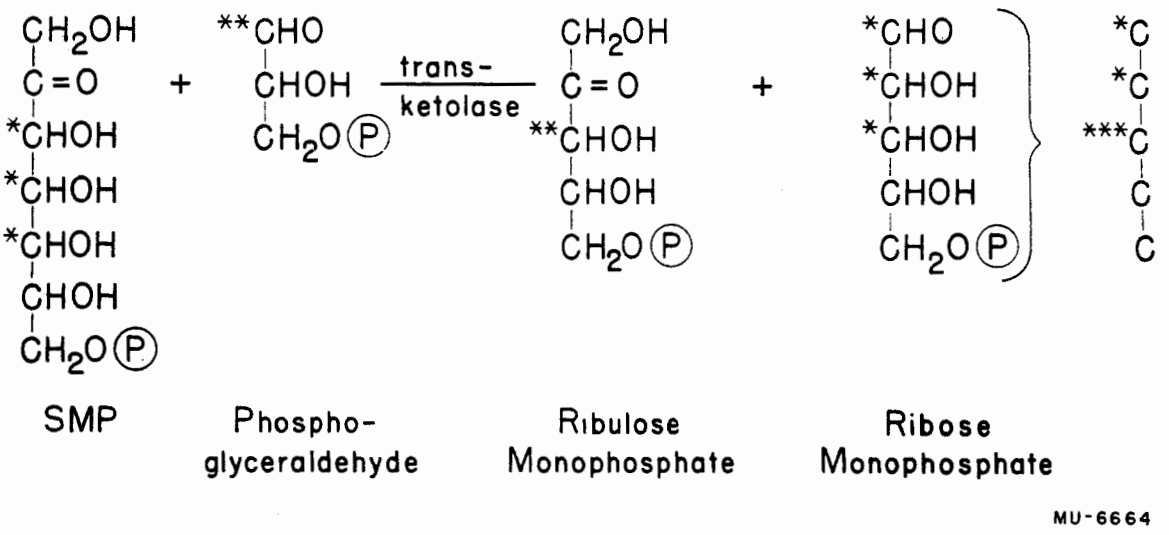

Fig. 9 Formation of 5-Carbon Sugars From Sedoheptulose Phosphate

them onto a three-carbon piece labeled as is phosphoglyceraldehyde, we would get two five-carbon pieces -- one ribulose and one ribose --and their label would be thus distributed. The average of them would be the actual one found. This, then, gave us a clue to the origin of the ribose and ribulose phosphates that we were finding -- that is, by a transketolase reaction of the sedoheptulose phosphate with the triose phosphate to give the two pentose phosphates. These can be interconverted by suitable isomerization. Thus, we have the pentoses formed from heptose and triose.

We know how the hexose is formed, from two trioses. The question then remains; where does the heptose come from? And here, again, a similar detailed and careful analysis was made of the carbon distribution within the heptose molecule as a function of time -- and this was a long-term job. It finally led to the realization that the heptose must have been made by the combination of a four-carbon with a three-carbon piece. And the question arose then: where does the properly labeled four-carbon piece come from? It could only come by splitting the C_6 (hexose) into a C_4 and a C_2. Figure 10 shows how this scheme was arrived at. Here are shown the two trioses, which as you know can make a hexose; one triose can react with a hexose to form a pentose and, under the influence of transketolase, a tetrose; the tetrose then can react with another triose, under the influence of the enzyme aldolase, to form the heptose, and give the proper distribution of carbon. So much, then, for the relation between the various sugars -- that is, the triose, the tetrose, the pentose, the hexose and the heptose. We have relationships between all of them and have formed them all from PGA, but not any of them yet has been marked--identified -- as the precursor that can accept the CO_2.

Identification of CO_2 Acceptor

The clue to this identification was obtained from an entirely different kind of experiment. It came, not from an experiment on the rate of appearance of radiocarbon such as the ones I have just described, but from an experiment in which the levels of the various compounds was measured--the actual total amount of each compound in the plant was measured-- and then determining the way in which that total amount changed, under changing conditions: For example, the amounts of pentose and triose and PGA (that is, the acid from triose) and a variety of other things, were determined in a steady state. Then we turned the light off to see what would happen to the various compounds. The results are shown in Fig. 11. It is clear here that in about five minutes enough carbon has passed into the plant to saturate certain of the compounds -- PGA, RuDP, HMP (hexose monophosphates) -- which are therefore functioning in some sort of cyclic operation, and to bring them up to the specific activity of the carbon dioxide entering the plant. The sucrose, which is a storage product, is, of course, not saturated -- it keeps getting more and more radioactivity. Immediately the light is turned off, a very interesting phenomenon occurs. The ribulose diphosphate -- the pentose phosphate -- drops precipitously, whereas the PGA increases very sharply. This immediately puts the finger on the RuDP as the immediate precursor to the PGA and suggests a cyclic system such as is shown in Fig. 12 as a possible scheme for the relationship between th RuDP, PGA, triose phosphate, and other sugars. Here it is the RuDP which, upon carboxylation with CO_2, gives PGA; we will write the details of that reaction later on. The PGA is reduced by reducing power (hydrogen) which is made by the photolysis of water,

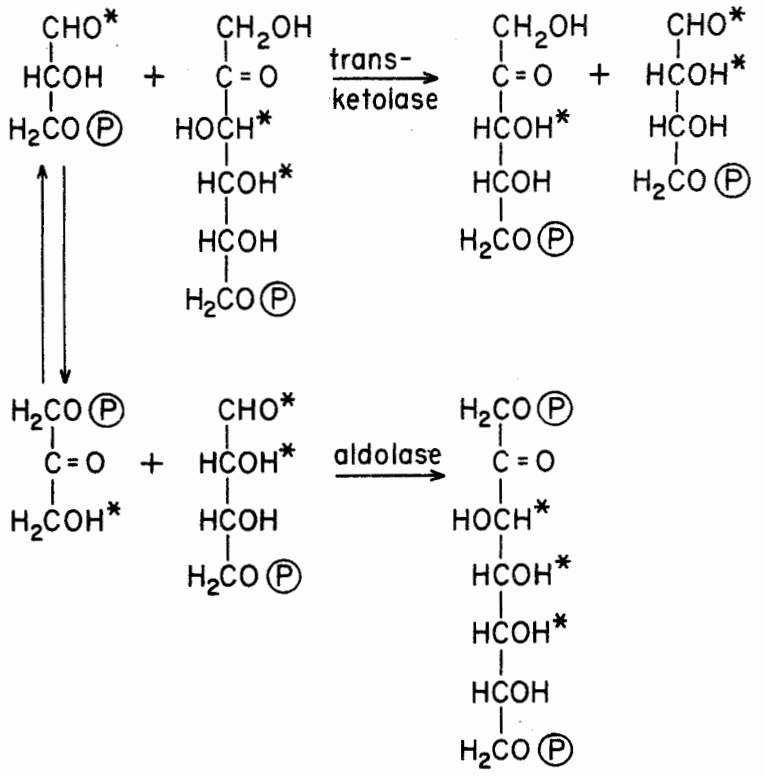

Fig. 10 Formation of a Heptose From Triose and Hexose

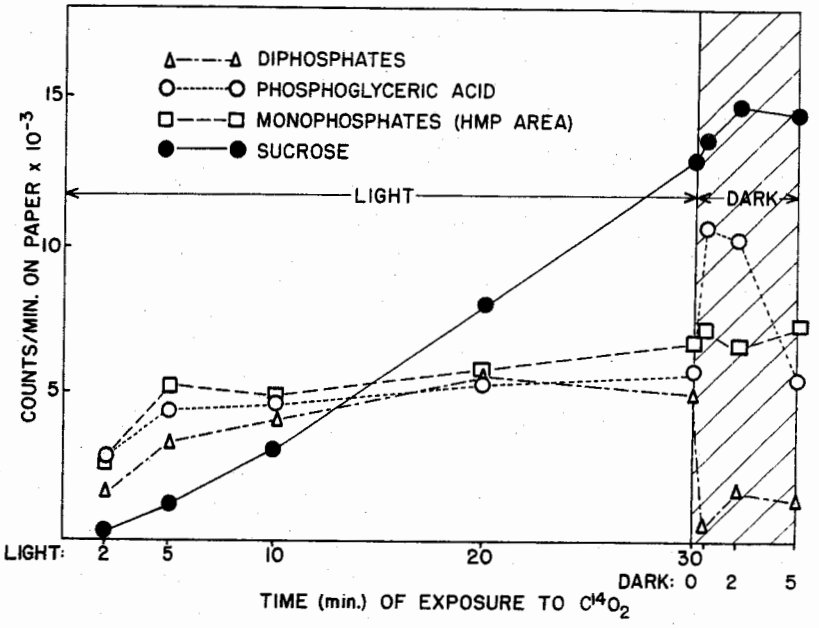

Fig. 11 Effect of Light and Dark on Concentrations of Phosphates and Sucrose

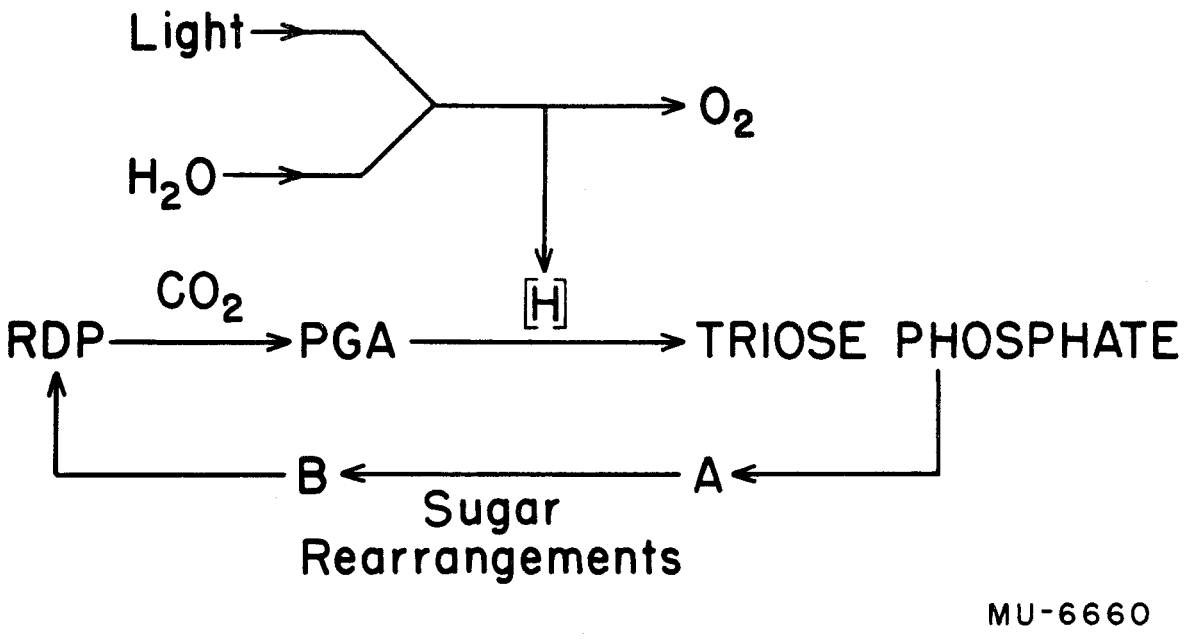

Fig. 12 Suggested Cyclic Scheme for Relationships in Photosynthesis

to triose phosphate (at the sugar level), the triose phosphate then undergoes a series of rearrangements, such as the ones described earlier, through the hexose, pentose, and heptose, back again to the ribulose. This is all at the sugar level of oxidation and requires very little energy for its operation. We thus are able to regenerate ribulose from triose, and it is clear that when we turn out the light, thus stopping the supply of reducing agent, the PGA should build up if CO_2 is present and RuDP should drop. This is precisely what we saw. In fact, it is the reason for drawing this particular scheme.

There is another deduction which can be made now from this scheme, before the experiment is done -- that is what would happen if the CO_2 pressure were suddenly changed. Again, you can see, it is easy to predict that if we suddenly reduce the amount of CO_2 present, keeping the light constant -- in other words, block the carboxylation reaction -- we should build up RuDP and lose PGA. And, indeed, that is what happens, and Fig. 13 shows this result. Here, again, is shown the cyclic scheme of Fig. 12 together with the sequence of events in its operation. At the time when the CO_2 drops from 1% to 0.003%, the first thing to rise is the RuDP, the next thing is RMP (ribulose monophosphate), and the last thing to rise is triose phosphate. The first thing to fall on the "forward pulse" is the triose phosphate; the next thing to fall is the RMP, and the last thing to fall is the RuDP. Thus we have a pretty good confirmation for the operation of such a cyclic feedback system.

THE CYCLE

Figure 14 shows the whole scheme put together with the details of the structural formulas and enzymes involved. Carbon dioxide enters via the formation of PGA by a carboxylation reaction on RuDP. This has been a postulated reaction required by the mechanics and kinetics of the system as we have so far described them. It has not until now been described as an isolated enzyme, as has each of the remaining reactions in the cycle. These are: triose phosphate dehydrogenase gives triose phosphate; the triose then undergoes the various transformations -- via isomerase to the ketose, which, the aldolase, makes the fructose diphosphate; with phosphatase to fructose monophosphate, then with transketolase to give the tetrose phosphate, which, again with aldolase, gives the heptose diphosphate; with phosphatase to the heptose monophosphate; with transketolase again to give both pentose phosphates; ribose phosphate via isomerase gives ribulose monophosphate, followed by a phosphorylation, using adenosine triphosphate (ATP) and phosphopentokinase to produce, finally, ribulose diphosphate. Now each of these enzymes from PGA through all these transformations has been prepared and described. Each of these reactions had already been carried out independently in solution, except for the first (or last) one involving CO_2.

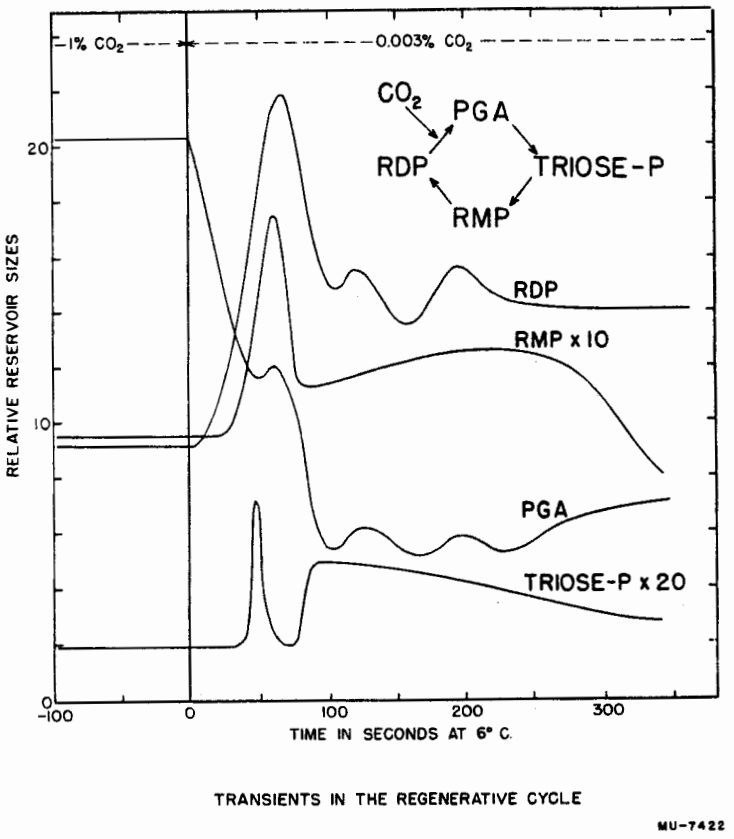

Fig. 13 Transients in the Regenerative Cycle

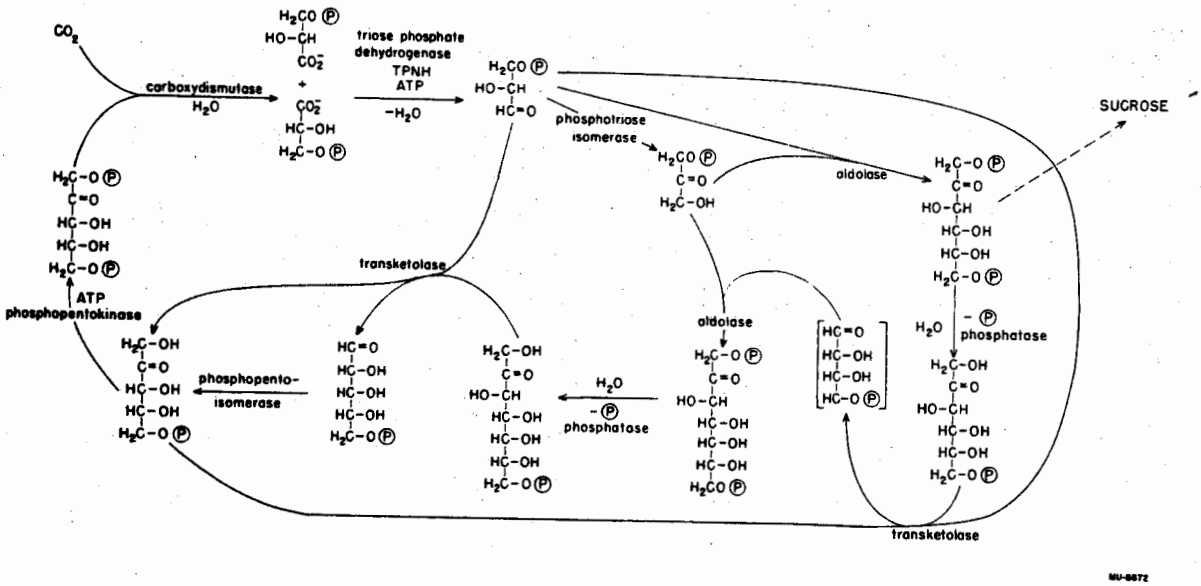

Fig. 14 The Complete Photosynthetic Carbon Cycle

Carboxydismutase

About a year ago now, directed by our tracer studies, we sought and found a cell-free preparation, both from algae and from other green plants, which was capable of catalyzing the production of PGA specifically from ribulose diphosphate (RuDP) and sodium bicarbonate. The RuDP used in these experiments was isolated by chromatography from green plant extracts. The technique was to expose the RuDP and the enzyme preparation to $NaHC^{14}O_3$ and show that carboxyl-labeled PGA was formed (Fig. 15). The traces of malic, citric, and aspartic acids, and alanine formed indicate the presence of some Krebs-cycle enzymes in the preparation as well, which could carry some of the PGA initially formed on to other things. Indeed, upon longer exposure (>three minutes) to these crude preparations, much of the PGA was converted. The formation of a little labeled malic acid in the absence of substrate (RuDP) indicates the presence of pyruvic acid and malic enzyme.

Since in this experiment the tracer was in the CO_2 and not in the RuDP, it did not give direct information about the fate of the five carbon atoms of ribulose. It was therefore necessary to do the experiment with labeled RuDP and unlabeled CO_2. This was not very satisfactory in the first instance when the crude preparation was used. Although labeled PGA was formed, a good many other labeled compounds were formed as well because of the presence, in the preparation, of enzymes that could act on ribulose diphosphate and things formed from it; in particular there was present a phosphatase which permitted the formation of ribulose-5-phosphate. This, in the presence of transketolase and aldolase (and possibly transaldolase) would rapidly find its way into hexose, heptose, and triose, the last of which might have given rise to some PGA by oxidation. While attempts to bypass this difficulty by inhibiting the initial phosphatase reaction on RuDP were partially successful, they were not conclusive, because of the insensitivity of the RuDP $\xrightarrow{HCO^-_3}$ PGA system to fluoride ion (F^-). It was therefore necessary to proceed with the attempt to free the preparation from any and all other enzymes capable of acting upon RuDP except the one (s) required for the PGA-forming reaction (from CO_2). This was accomplished first from neutral extracts of New Zealand spinach (Tetragomia expansa), and later from extracts of sonically ruptured algae. The enzyme appears in the protein fraction, salted out of neutral extracts, between approximately 0.3 and 0.4 of saturation with $(NH_4)_2SO_4$. The results of an early experiment with such a preparation acting on labeled RuDP are shown in Fig. 16[1] Here the fate of the ribulose carbon is clearly in PGA when both enzyme and $NaHCO_3$ were present. There appears to be some sugar monophosphate present in all the experiments, partly because of its presence in the original RuDP sample and perhaps partly because of the presence of some residual phosphatase in the enzyme preparation. Later experiments have given preparations that convert essentially all of the ribulose carbon into PGA and nothing else.

It thus appears that the original formulation of the reaction is at least

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(1) Unpublished experiments in this laboratory by J. Mayoudon, I.R.S.I.A. Fellow, Brussels, Belgium, 1954.

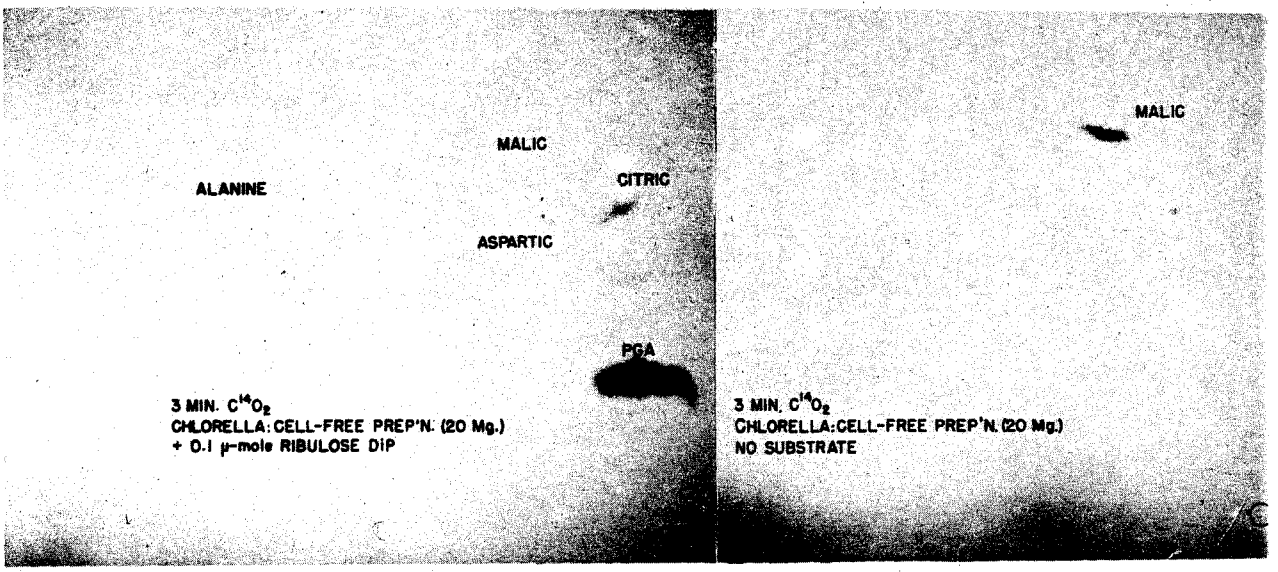


Fig. 15 Chromatograms Indicating Formation of Carboxyl-Labeled PGA

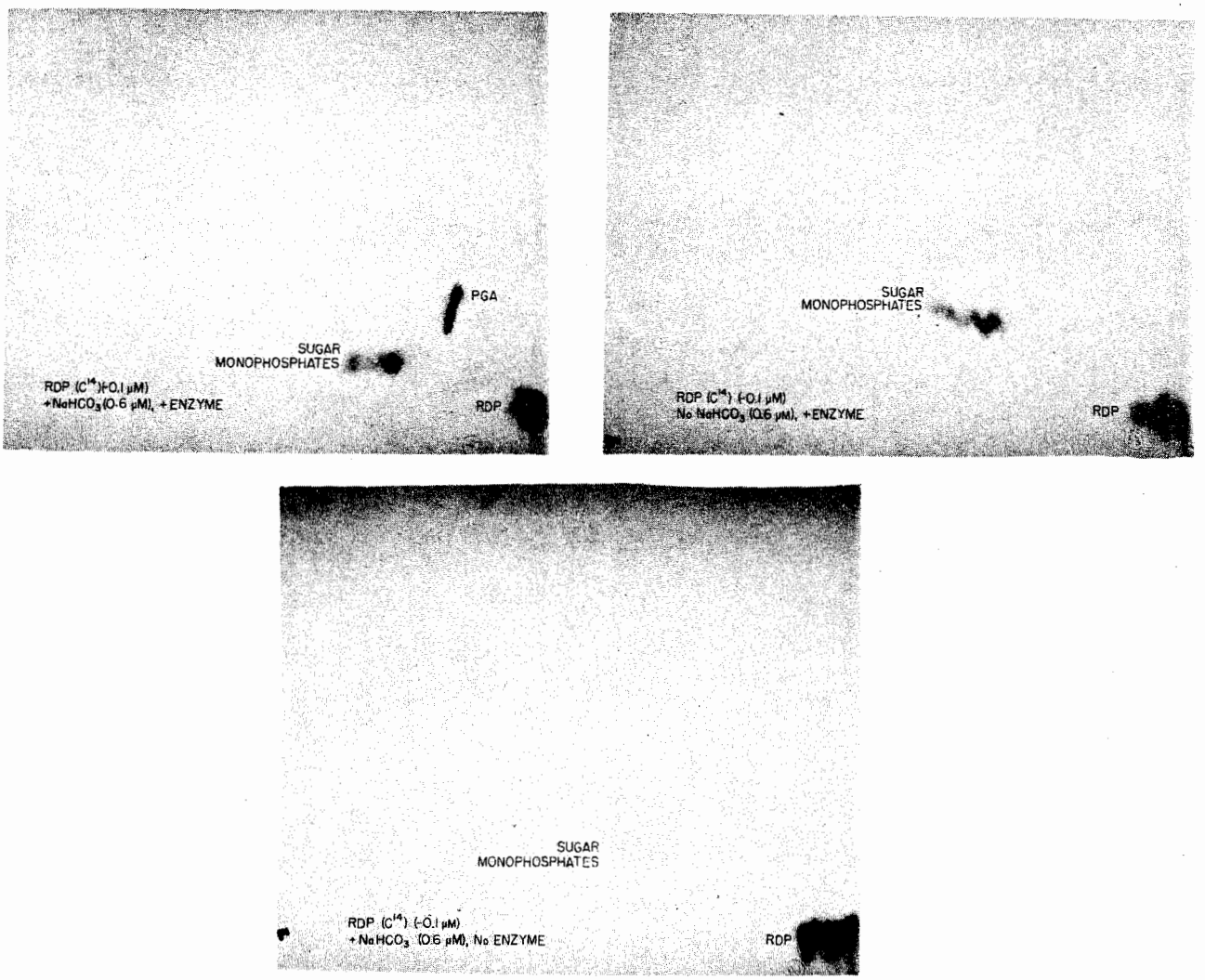
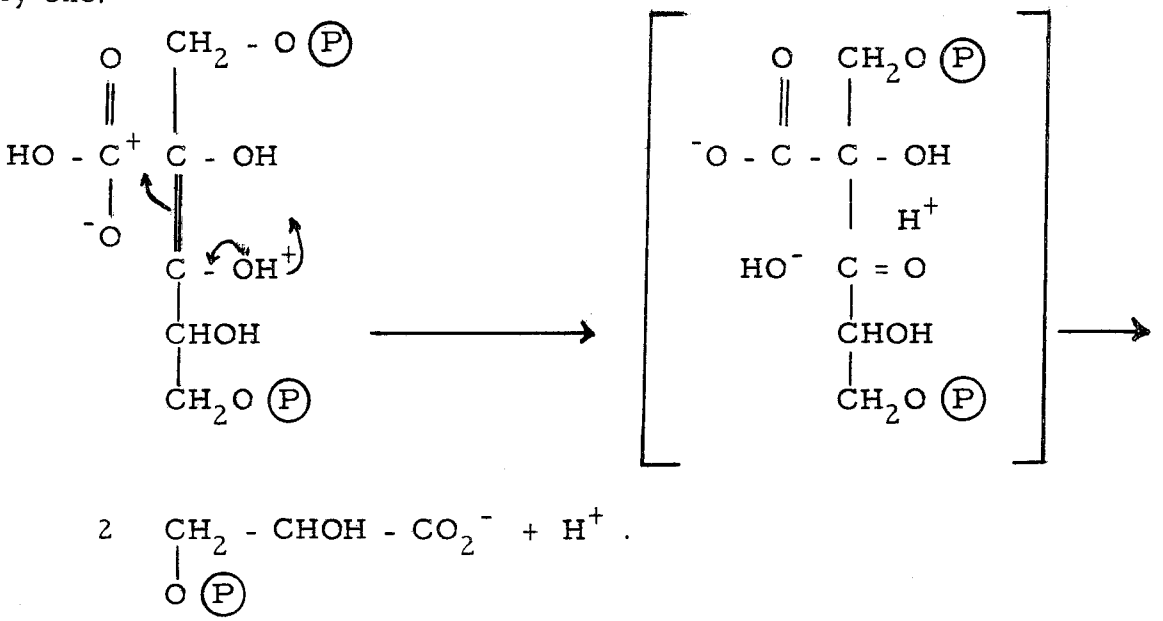


Fig. 16 Chromatograms Showing Effect of Enzyme Action on Ribulose Diphosphate

a likely one:*



2 $CH_2 - CHOH - CO_2^- + H^+$.
$O(P)$

Because the carboxylation reaction takes place at the expense of the oxidation of C_3 of the ribulose to the carboxyl level, the name "carboxydismutase" suggests itself as uniquely descriptive. It is interesting to note that the enzyme is not readily demonstrated in animal tissues (rat liver), and that it can be obtained from spinach in association with the highly organized intact chloroplasts,[1a] from which it is extremely easily separated. It does not appear to be especially sensitive to versene, o-phenanthroline, or cyanide, but it is sensitive to p-chloromercuribenzoate, which inhibition is reversed by cysteine.

Chemical Requirements to Run the Cycle

Now to return to the scheme of things again. We now have the cycle in its details (Fig. 14), and we know now precisely what reagents are required to make the cycle turn. Let us have a look at the reagent requirements to keep this cycle running, and see if we can determine something about the energy requirements for it. You can see here that what is required for the reduction of a PGA molecule to a triose is one molecule of triphosphopyridine nucleotide (TPNH) and one molecule of adenosine triphosphate (ATP). There is no further energy requirement in any of the sugar rearrangements, until we get to the point of ribulose-5-phosphate, where we require again another molecule of ATP to make the RuDP. A calculation of what is required per CO_2 molecule entering will show that the net requirement for the production of triose phosphate, that is, the net reduction of one molecule of CO_2 to the carbohydrate level, is four equivalents of reducing agent -- that

(*) A possibility remains that, in the light, reductive fission between C_2 and C_3 of the carboxylated pentose (enzyme-bound) might take place as an alternative to the known hydrolytic cleavage shown above, and give rise to only one molecule of PGA and one molecule of phosphoglyceraldehyde. At present the evidence seems not to favor this.

1a R. Clinton Fuller, unpublished observations in this laboratory.

is, four electrons and three molecules of ATP. Thus, at least as far as the CO_2 is concerned, we have the complete energy requirement established. For each molecule of CO_2 that goes to carbohydrate we have to supply four electrons -- four equivalents of reducing power -- and three molecules of ATP. All of this must be made ultimately by the light -- by the conversion of the electromagnetic energy in some way. You will notice that in this requirement for reducing carbon there is no particular requirement for a photochemical reaction other than the production of the two reagents. If we could supply those two things from some other source than the photochemical reaction, we should be able to make this whole sequence of operations function. We have reason to believe that this is indeed being done by the use of the required collection of enzymes. But a suitable situation exists in nature also. The situation is such that we must have simultaneously a high level of this particular reducing agent, which we now know can be triphosphopyridine nucleotide (TPN), and ATP at the same time and the same place.

Running the Cycle Without Light

There is one system in nature, aside from the green plants, in which that situation occurs. At least we know about this one; it is in one of the photosynthetic purple bacteria that don't make oxygen, but do reduce carbon dioxide with molecular hydrogen. Figures 17 and 18[2] show that it is possible to have the reduction of CO_2 take place either through the agency of light or through the agency of a chemical oxidation system. The organism is the purple bacterium, Rhodopseudomonas capsulatus. The initial slope corresponds to the reduction of carbon dioxide in the light; this is using hydrogen as the reducing agent, but light is required also. As soon as the light is turned off, the reduction of carbon dioxide stops. Figure 18 shows the same organism. This is a dark fixation. Here it is exposed only to helium and hydrogen, and there is an initial fixation which immediately saturates and then stops; but then when oxygen is admitted to the system, the fixation again continues in the same way as it does with light. The intermediates in the dark are very much the same. The hydrogen is presumably there to provide the reducing power that is needed. The oxygen is required to oxidize some of that hydrogen to make ATP, and the two together, then, can make the carbon dioxide cycle function. This suggests that a prime function of the light, when the high reducing potential of hydrogen is the reducing agent, is to supply the oxidizing agent necessary for the production of the required ATP.

Quantum Requirements

In order to estimate what a minimum quantum requirement for photosynthesis may be on the basis of the information we have so far accumulated about the detailed chemistry of the process, at least one assumption is necessary, relating to the mode of interaction of electromagnetic radiation and matter. It is that a single quantum can excite not more than a single electron. Another assumption about the behavior of the excited electron is required, namely, that it does not by some chemical (or physical) dismutation process give rise to more than one equivalent of reducing power at the potential of TPNH. And if that is the case, then, just from looking at the requirements we mentioned a few moments ago, one can predict what the minimum quantum requirement for such an operation, not counting the efficiency of the oxygen-evolution end of the system, would be. We need the four electrons for the

(2) A. O. M. Stoppani, R. C. Fuller, and M. Calvin, J. Bact. (in press).

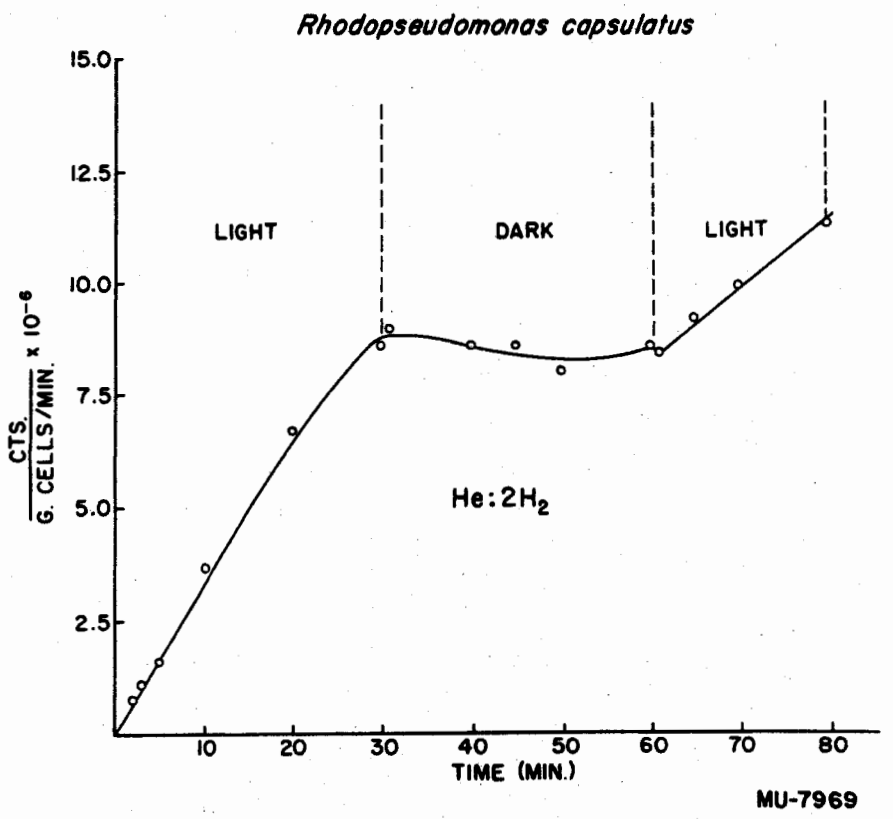


Fig. 17 Photoreduction of CO_2 by Purple Bacteria

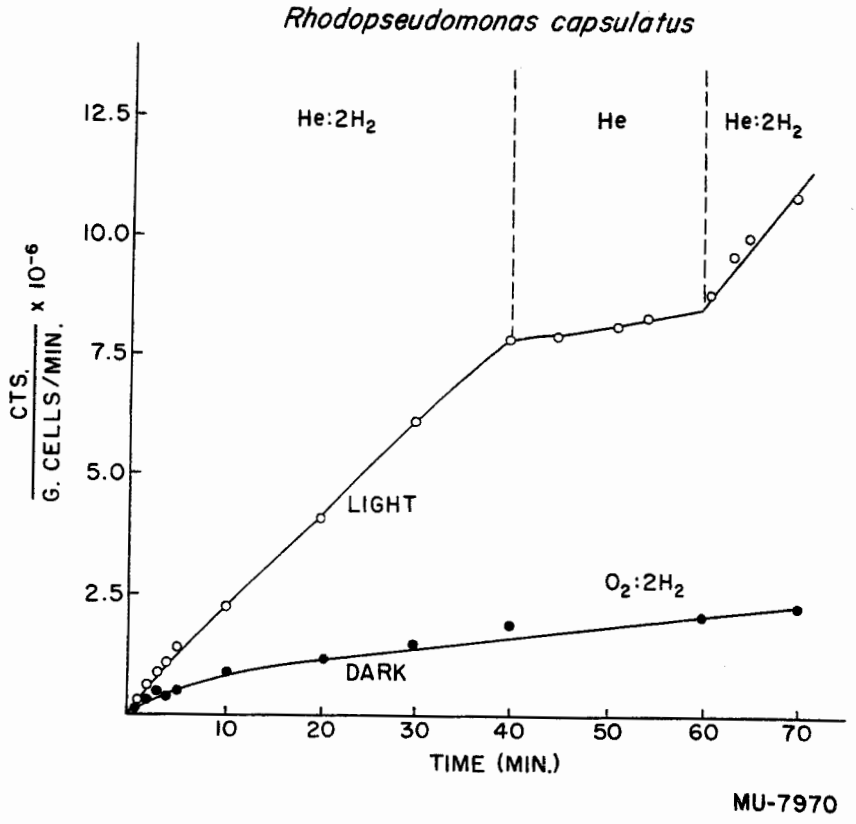


Fig. 18 Chemical Reduction of CO_2 by Purple Bacteria

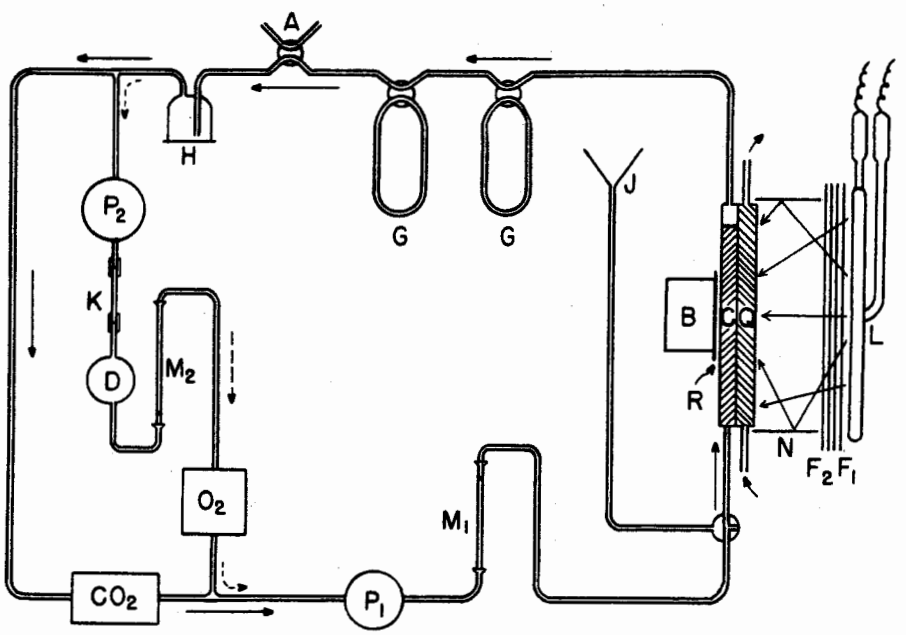


Fig. 19 Schematic Diagram of Apparatus for Measuring Quantum Requirement of Photosynthesis. C, Algae Vessel; B, Bolometer; Q, Water Jacket; N, Reflecting Tube; O_2, Oxygen Analyzer; CO_2, Carbon Dioxide Analyzer; A, Stopcock; K, Capillary, P_2, Pump; D, Bulb; G, Tubes Containing N_2; H, Rubber Membrane; J, Inlet for Introduction of Algae; F, Filters, M, Flow Meters; R, Opal Glass

reduction, and we need three molecules of ATP.

Now we already know something about the various ways in which ATP can be produced. We know, for example, that by the transfer of two electrons from DPNH to an atom of oxygen -- one-half mole of oxygen -- we can produce approximately two or three molecules of ATP. Therefore one can suppose, on this basis, that the minimum quantum requirement, when all the energy for the operation of this cycle comes from light and only from light ultimately must be six -- that is, four electrons for the reduction and two more for the three molecules of ATP that are required. It should be possible, however, to find conditions under which the quantum requirement for the reduction of CO_2 and the evolution of oxygen would be as little as four, provided there were some other source besides the light for the three molecules of ATP. And indeed these conditions we have realized. This was an incidental work.[3] The quantum-requirement determination was quite accidental. We happened to have the apparatus set up in which one could measure directly, without any ambiguity, the production of oxygen by a direct measurement of some unique quality of the oxygen -- not merely a gas pressure -- and this quality happened to be the paramagnetism. Also we could measure directly the amount of carbon dioxide absorbed, by measuring some property of the CO_2 in the gas phase, not merely its pressure -- and this happened to be its infrared spectrum. Between the two of these, by direct measurements, then, of the gases evolved and absorbed under the influence of light in a system in which the light absorbed could be directly determined without too much complication in terms of scattering -- a very flat system -- we were able to measure the quantum requirement for oxygen production under a variety of conditions. Figure 19 shows the type of equipment that was used. Figure 20 shows the kind of data that you get when the output of the two analyzers is fed into a multipoint recorder. The lines correspond to the CO_2 and O_2 partial pressures.

From the volume of the system we can calculate exactly how many moles of each gas are being absorbed, or emitted, per minute. From the light absorbed we can calculate the quantum requirement, and this result is shown in Fig. 21. Here the solid points give the apparent quantum requirement for net oxygen evolution without any correction whatever. This is assuming nothing; it is simply the number of molecules of oxygen evolved divided by the number of quanta of light absorbed in the same time. And you see that at very high light intensity -- q is the number of quanta absorbed per second -- the quantum requirement drops down to something less than 8 (about 7, or 7.5 or thereabouts). The P/R scale is the ratio of the rate of photosynthesis to the rate of respiration in the dark period immediately following that photosynthetic measurement. In the high-intensity range the rate of photosynthesis was ten to twelve times that of the reverse reaction, that is, the absorption of oxygen in the dark period immediately following. If we assume -- and we have every reason to believe this is so -- that the oxygen absorption in the light period just preceding is going on at very nearly the same rate as it does when you turn off the light and this rate of reabsorption is added to the net evolution of oxygen, then the quantum requirement falls to that corresponding to the open points. As the amount of of respiration with respect to the photosynthesis gets larger and larger, the

(3) J. A. Bassham, K. Shibata and M. Calvin, Biochem. Biophys. Acta, (in press).

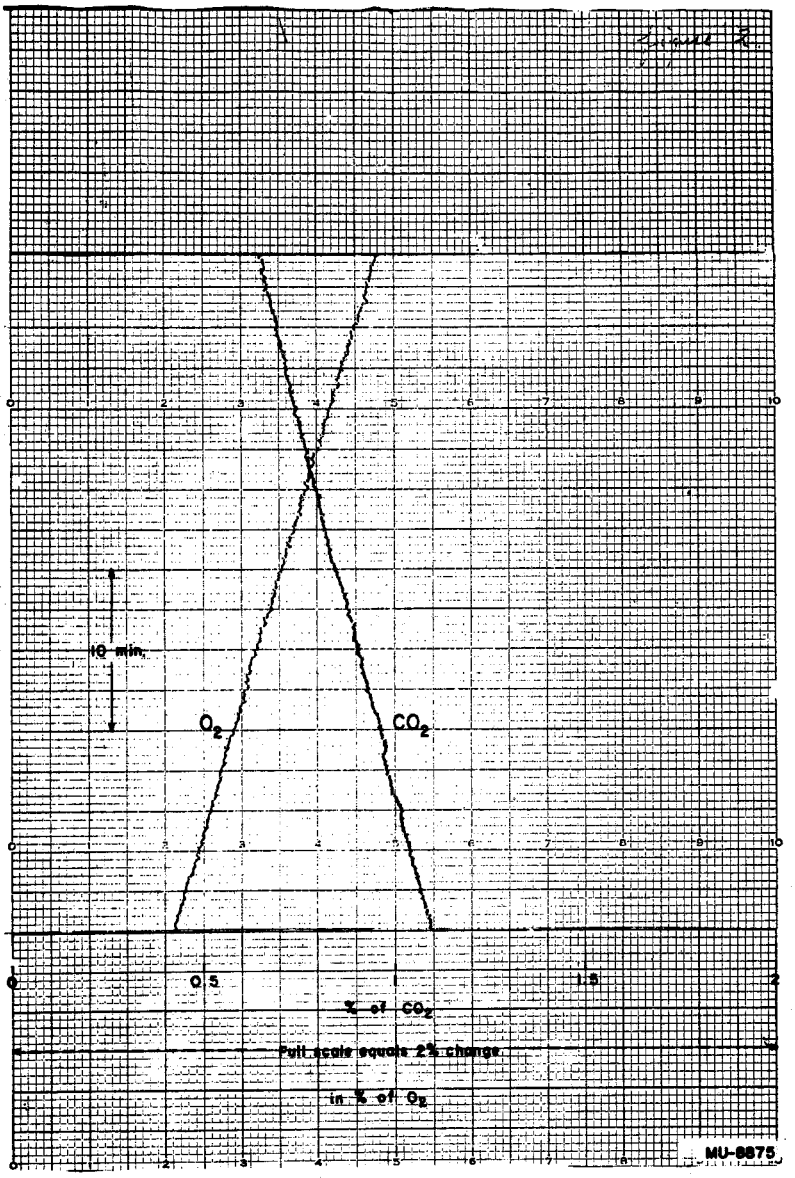


Fig. 20 The Directly Recorded Gas Exchanges in Quantumyield Determination

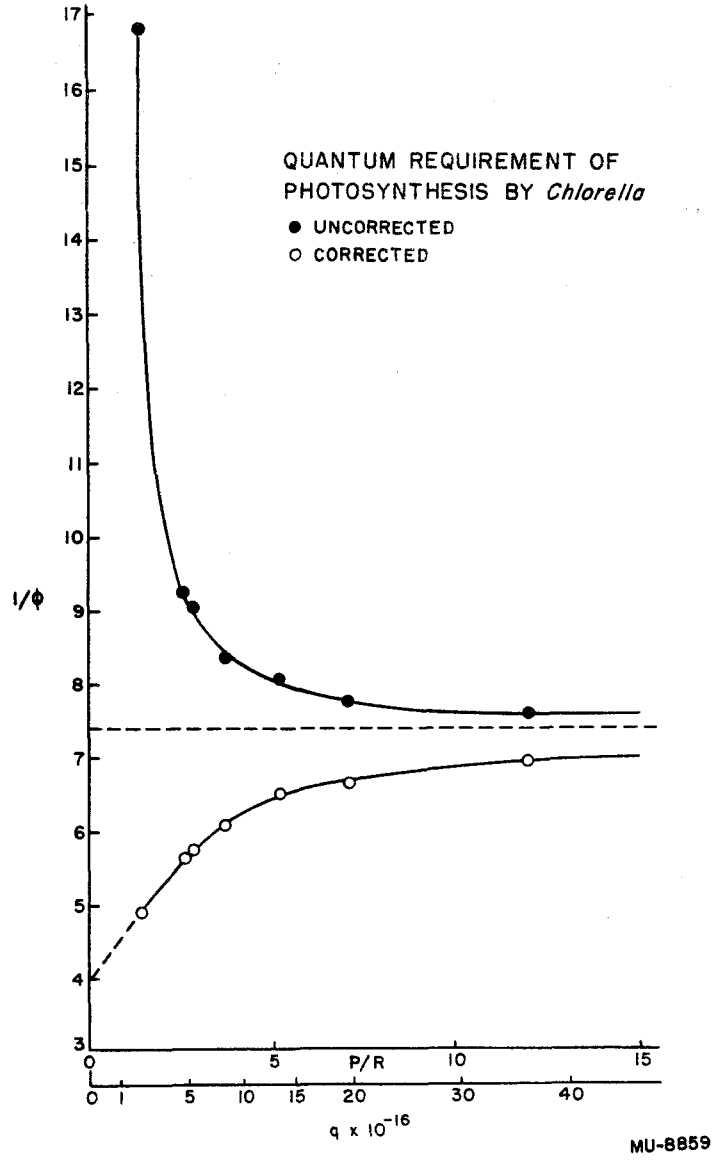


Fig. 21 Quantum Requirement of Photosynthesis by Chlorella

quantum requirement gets smaller and smaller. You see it extrapolates at low levels of photosynthesis to about four.* Under these conditions, the light is not called upon, or at least the electrons generated by the quanta are not called upon, to produce ATP; they can be used exclusively for the reduction process, and so the quantum requirement may be as low as four. But if the rate of reduction becomes so great that the rate of generation of ATP outside the chloroplasts is not fast enough to keep this up -- or at least the diffusion rate is not fast enough and ATP must be generated at the site of reduction -- then we must use some of those photoproduced electrons to make ATP, and the quantum yield will fall, or the quantum requirement will rise to something approaching seven.

This seemed like a very useful and good confirmation of the general notion that we have so far presented. I would like to tell you how the light makes the electrons and how the electrons can be converted into ATP when it is necessary -- but unfortunately I don't know yet. However, we do have some very stimulating indications and I will try to tell you about them.

QUANTUM CONVERSION

So far we have been talking only about the reduction of carbon. And because this seems to be quite a separate system from the oxygen-evolution reaction, it would appear that we shouldn't expect to learn a great deal about the photoproduction of the electrons and the ATP from studying the carbon reduction. But it so happens that there is -- and obviously there must be -- a connection between the two, and that connection is fairly close. By suitable observations we were able to see at least one point at which the carbon-reduction cycle makes contact directly with the photochemical apparatus. This is shown in Fig. 22. Here is the cycle again. The quantum is first absorbed by chlorophyll and converted into something making a reducing agent [H] and some oxidizing agent [O] ; the reducing agent can reduce the glyceric acid to triose. Some of the reducing agent must be used to make ATP, with oxygen or the intermediates on the way to oxygen, because that is necessary for the cycle to run; this is what we have already talked about. What we are going to talk about now is this point of contact, [H] , between the photochemical apparatus and the carbon cycle and what information we can learn about the quantum conversion part from studying this.

Light Inhibition of TCA Cycle Incorporation

The experiment that was done was one in which a steady state was examined and the changes induced by a sudden change of conditions were observed. Figure 23 shows this. Here we have the same type of experiment as before, but we are looking at different substances now. Focus your

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(*) This value of four as the quantum requirement at low photosynthetic rates is in no way comparable to the values between three and four reported by Warburg and his associates at very high P/R ratios (> 20).[4]

(4) O. Warburg, G. Krippahl, W. Buchholz, and W. Schroder. Z. Naturforsch. 8b, 675 (1953).

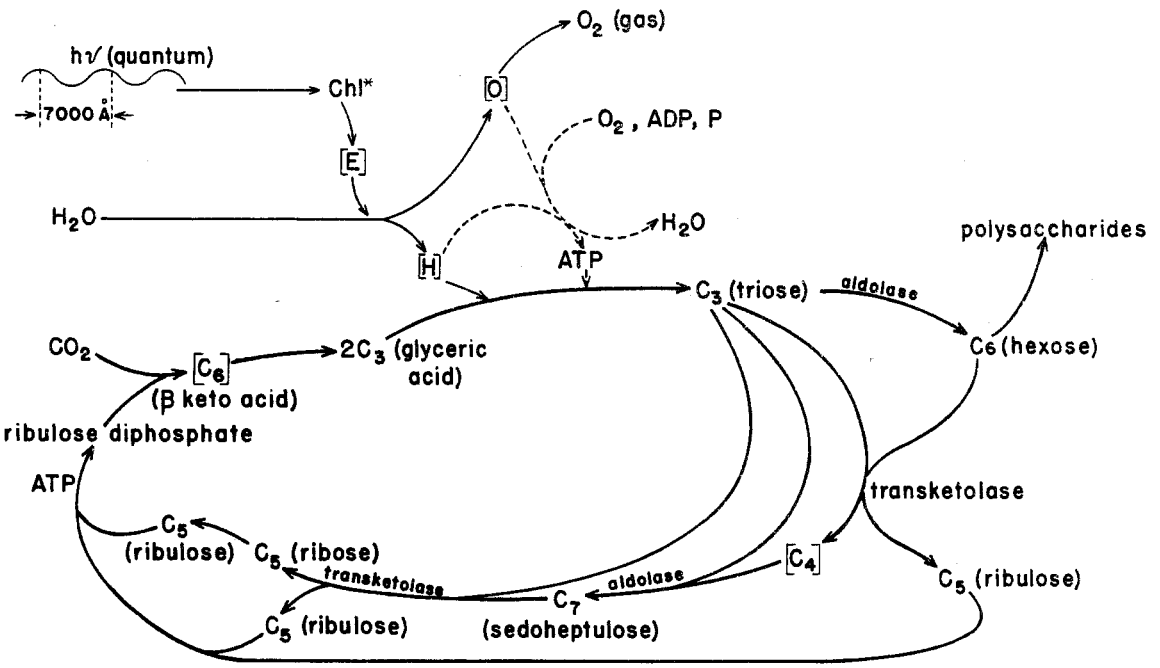

Fig. 22 Proposed Cycle for Carbon Reduction in Photosynthesis

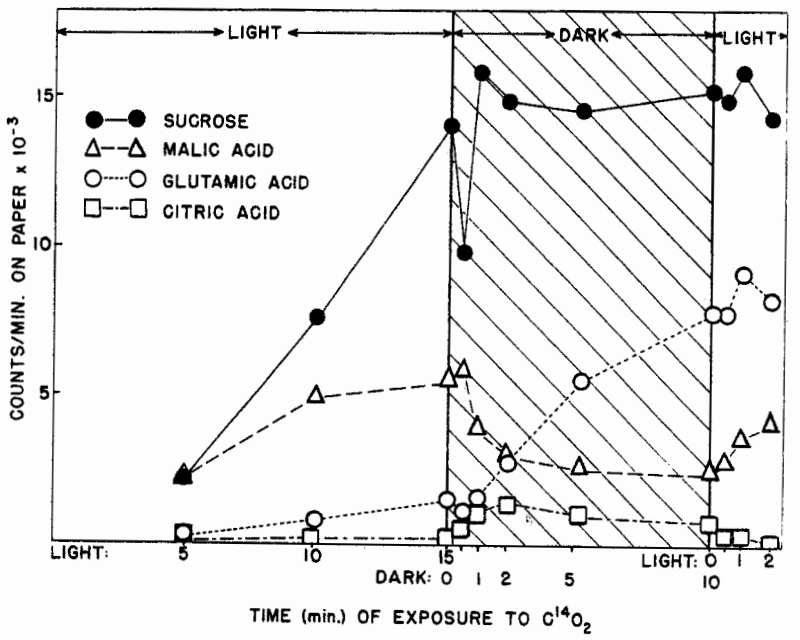

Fig. 23 Effect of Light and Dark on Formation of Sucrose, Malic Acid, Glutamic Acid, and Citric Acid

attention particularly on glutamic acid and citric acid and you will notice that while the light is on, the rate of formation of radioactive glutamic acid and radioactive citric acid is quite low. But immediately the light is turned off, the rate of formation of radioactive glutamic acid is increased many-fold; the rate of formation of radioactive citric acid also increases many-fold. Now glutamic and citric acids are two compounds very closely related to the respiratory cycle known as the Krebs cycle, and Fig. 24 describes in schematic terms the experimental facts we have just seen. Here is shown the photosynthesis cycle with which you are already familiar. Here also is the Krebs -- or tricarboxylic acid -- cycle, the details of which are shown in Fig. 25. The glutamic acid and citric acid are in or related to the Krebs cycle. The photosynthetic cycle, you already have seen, and it does not contain either glutamic or citric, but it does, as you know, form PGA and the sugars. Eventually these direct products of the photosynthesis cycle have to get into carbohydrates and proteins and fats, and ultimately they will get back into the tricarboxylic acid cycle. That is the major route in the light. But immediately the light is turned off a direct connection between the two cycles is apparently opened up, which allows the PGA to go over directly into the compounds of the tricarboxylic acid cycle. Figures 25, 26, 27 show the details of that mechanism. Figure 25 shows the tricarboxylic acid cycle and how the carbon can get into that cycle via acetyl Coenzyme A, condensing with oxalacetic acid to citric acid, which is one of the compounds we talked about, going around this route and leading over to glutamic acid. The question is: how does glyceric acid get to acetyl Coenzyme A? This must happen rapidly only in the dark, not very rapidly in the light. Fortunately we have some idea how acetyl-CoA may be formed from glyceric acid, and Fig. 26 shows this. The glyceric acid is dephosphorylated to form pyruvic acid; the pyruvic acid then reacts with an enzyme system of which thioctic acid is a coenzyme to form acetyl-thioctic acid and carbon dioxide. The acetyl thioctic acid then undergoes a thiol ester interchange with CoA to form reduced thioctic acid and acetyl-CoA, which then goes on into the citric acid cycle in the way shown in Fig. 27.[5]

Now, how does light affect this? This reaction (Fig. 26) is the door for the entrance of carbon into the tricarboxylic acid cycle, and if somehow we close this door by removing, or reducing the level of the disulfide, we can reduce the rate of appearance of radioactive carbon from the photosynthetic cycle into the citric acid cycle. This, then, suggests that the light shifts the equilibrium from the disulfide to the dithiol form by inducing reaction with something other than pyruvic acid, probably ultimately water. In the dark, oxidation brings it back again to the disulfide and the thing starts working again. This system is like a valve which is closed by light and which controls the flow of carbon from the photosynthetic cycle directly into the tricarboxylic acid cycle. It suggests further that the disulfide may be closely allied to, if not exactly, the electron acceptor from the photochemical act. Actually a number of experiments have been performed that indicate that this may be so.

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(5) J.A. Bassham and M. Calvin, "Photosynthesis", chapter in Currents in Biochemical Research, Interscience Publishers, Inc., (in press)

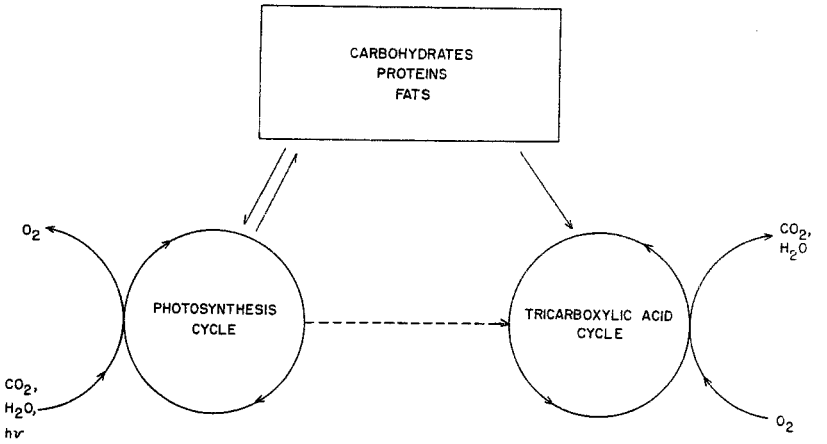
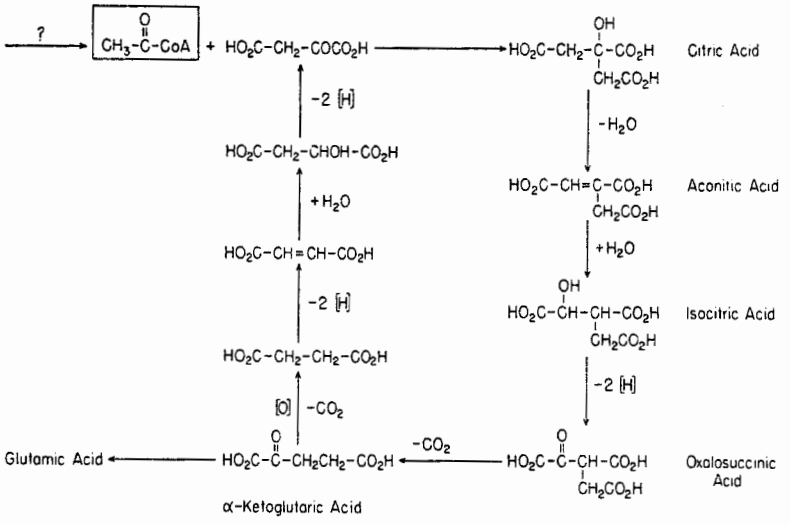


Fig. 24 Schematic Relationships Between the Photosynthesis Cycle, the Tricarboxlic Acid Cycle and Storage Products in the Plant



Tricarboxylic acid cycle

Fig. 25 Tricarboxylic Acid Cycle

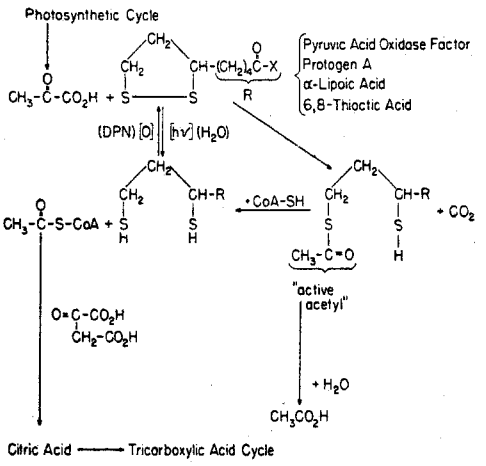


Fig. 26 Mechanism of Photochemical Control of the Relationships Between the Photosynthesis Cycle and the Tricarboxylic Acid Cycle.

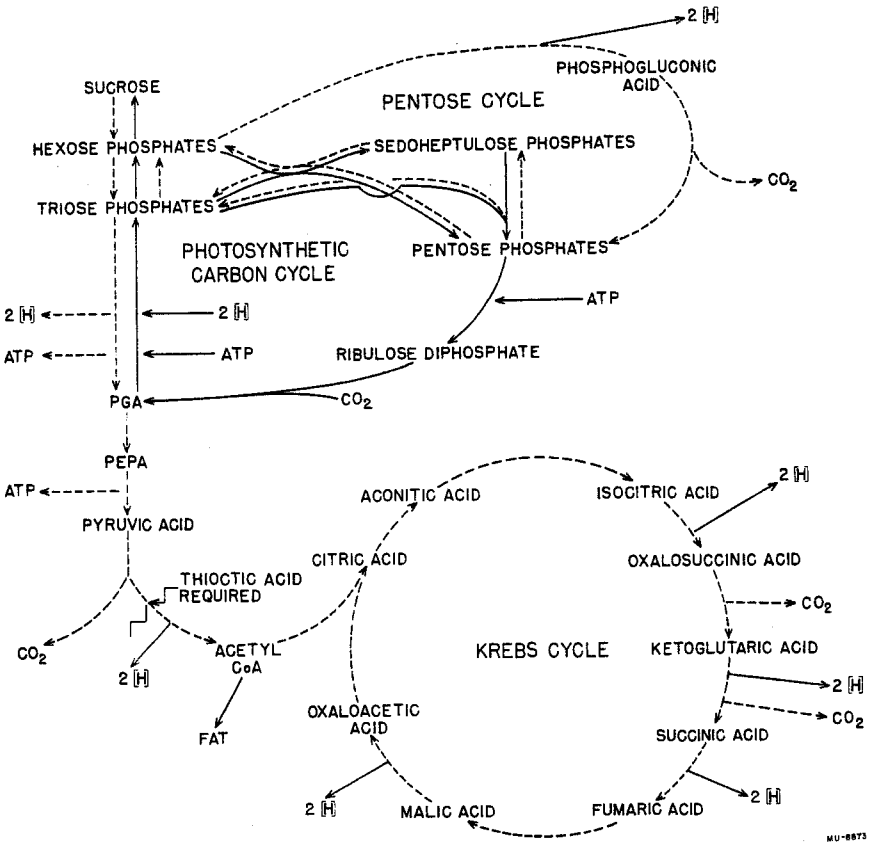


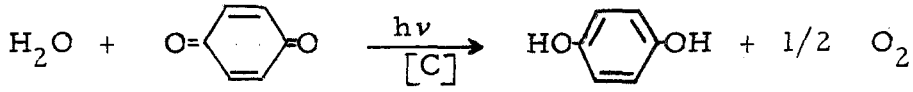
Fig. 27 Some Relationships Between the Photosynthetic Cycle and Respiratory Cycles; (1) Krebs Cycle and (2) Pentose Cycle

------ Oxidative Pathways

——— Reductive (Photosynthetic) Pathways

Thioctic Acid Effects

For some time now it has been possible to produce oxygen from water by illumination of grana, chloroplasts, or whole algae in the presence of some suitable electron acceptor (oxidizing agent) other than CO_2, for example, quinone (Hill reaction),



where [C] is a chlorophyll-containing structure, having some minimum size and degree of organization. If the thioctic acid (or some relative of it) is indeed as closely associated with the photochemical part as above suggested, it might be expected that its addition to a suitably limited system would increase the quantum yield for oxygen production. This has indeed been observed.[6] Although you could increase the quantum yield of oxygen production by adding thioctic acid, it could only be done under conditions under which the electron acceptor was rate-limiting.

Figure 28[6] shows the increase of the quantum yield by adding thioctic acid to a system that is producing oxygen; quinone is the electron acceptor. And it works only under conditions in which the electron acceptor is rate-limiting, so the thioctic acid can be the electron acceptor but does not have to be the acceptor of the oxygen of the water molecule. In fact, the differential increase in rate per mole of added thioctic acid may be ten times that for quinone, indicating its much greater efficiency for this process in spite of the fact that thermodynamically it is much the more difficult to reduce.

Although this indicates that thioctic acid can and does function between the light activation and the reduction of quinone, it does not answer the question of whether or not there is anything between the light activation and the reduction of thioctic acid. Thioctic acid acceleration experiments with flashing light[7] (~200-microsecond flashes), in which the dark time between flashes was sufficiently long so that no reaction not directly involving the photoactiviated state could be rate-limiting, failed to give an unequivocal answer, partly because a high enough intensity per flash to saturate was not reached. They did indicate, however, that with the concentrations of thioctic acid available[8] the lifetime of the electronically excited condition in the functioning chlorophyll-containing natural structure [C] should be quite long, longer than anything observed for chlorophyll in molecular solutions. It should be specifically stated that this requirement obtained only if the thioctic acid had to function directly in the mechanism of oxygen

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(6) D.F. Bradley and M. Calvin, Arch. Biochem. and Biophys. 53, 99 (1954).

(7) Dan F. Bradley, unpublished experiments from this laboratory.

(8) As determined by its biological activity for the growth of propionate-inhibited S. faecalis. R. Clinton Fuller, unpublished measurements from this laboratory.

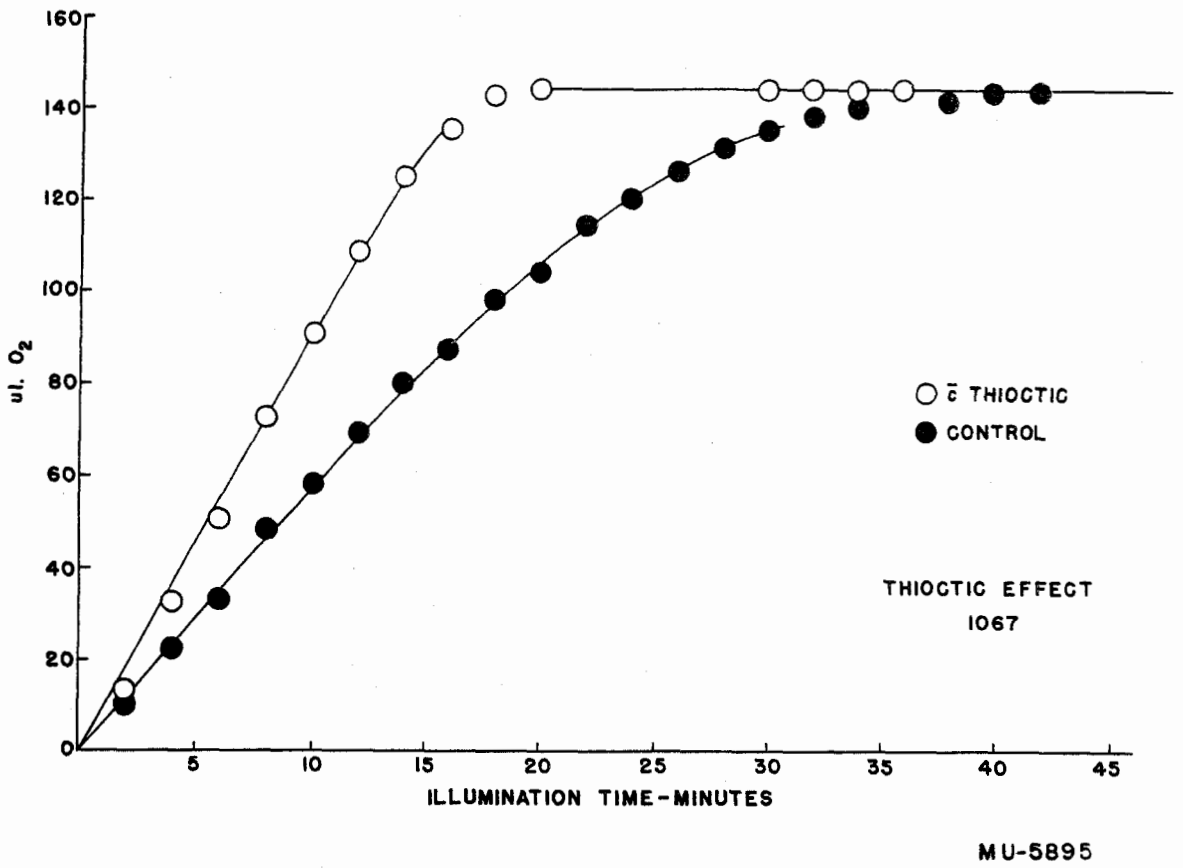

Fig. 28 The Thioctic Acid Effect on the Rate of Oxygen Evolution by Illuminated Algae in the Presence of Quinone (See Reference 6)

evolution as well as in the reduction part.[9] However, the original notion[10] of its function involved only its reduction to dithiol, i.e. as electron acceptor. This kind of separation of function is given further support by the apparent insensitivity to mercury poisoning of the Hill reaction with quinone,[11] as well as some other considerations. If therefore we give up the idea that the thioctic acid has to accept both the hydrogen and oxygen in the splitting of the water molecule, and consider that it has to accept only the hydrogen--that is, only the electron, with something else accepting the oxygen (or, if you like, taking the electron away from the oxygen)-- then the situation is relieved and we can retain the thioctic acid as the acceptor of excited electrons only. Something else has to be recognized as the electron donor, or, in other language, the acceptor of the positive hole left by the removal of the excited electron.

With this we arrive at a proposal which not only has precedent in physics and physical chemistry and resolves most, if not all, of the apparent difficulties we have so far recounted, as well as much information not mentioned here,[5] but also provides a function for the type of microstructure now becoming apparent (laminations on a macromolecular level) within the subunits (grana) of the chloroplasts.[12] It involves the basic notion that the chlorophyll functions photochemically as an organized, oriented aggregate, and not as individual molecules;[13] and that the absorption of light in this aggregate raises an electron from a molecular level to a conduction level. Figure 29[5] contains a schematic representation of this notion, together with its relationship to the photosynthesis cycle, the Krebs cycle and oxygen evolution, and the phosphorylation mechanism (ATP-producing system). The electrons and holes photocreated are immediately separated to opposite sides of a laminated structure by the presence of a built-in field, possibly such as exists at an n-p junction. The "electrode" reactions taking place are written out on either side of the triple-layer diagram (Figure 29b).

One of the major problems to which this suggestion provides an answer is this question of the lifetime of the excited state. You probably have heard many arguments leading to the final conclusion that because of the efficiency and kinetics with which the photosynthetic process proceeds, either the excited state must last for a very long time, or else the converting factor --

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(9) As suggested by J.A. Barltrop, P.M. Hayes, and M. Calvin, J.Am. Chem. Soc. 76, 4348 (1954).

(10) J.A. Barltrop and M. Calvin, J. Am. Chem. Soc. 74, 6154 (1952).

(11) J.S.C. Wessels and E. Havinga, Rec. trav. chim. 71, 809 (1952); 73, 1076 (1953).

(12) A. Frey-Wyssling, Endeavour, 14, 34 (1955).

(13) Resembling in this one respect the "photosynthetic unit" of R.B. Emerson and W. Arnold (J. Gen. Physiol. 16, 191 (1932), and Wöhl, New Phytologist 40, 31, (1941).

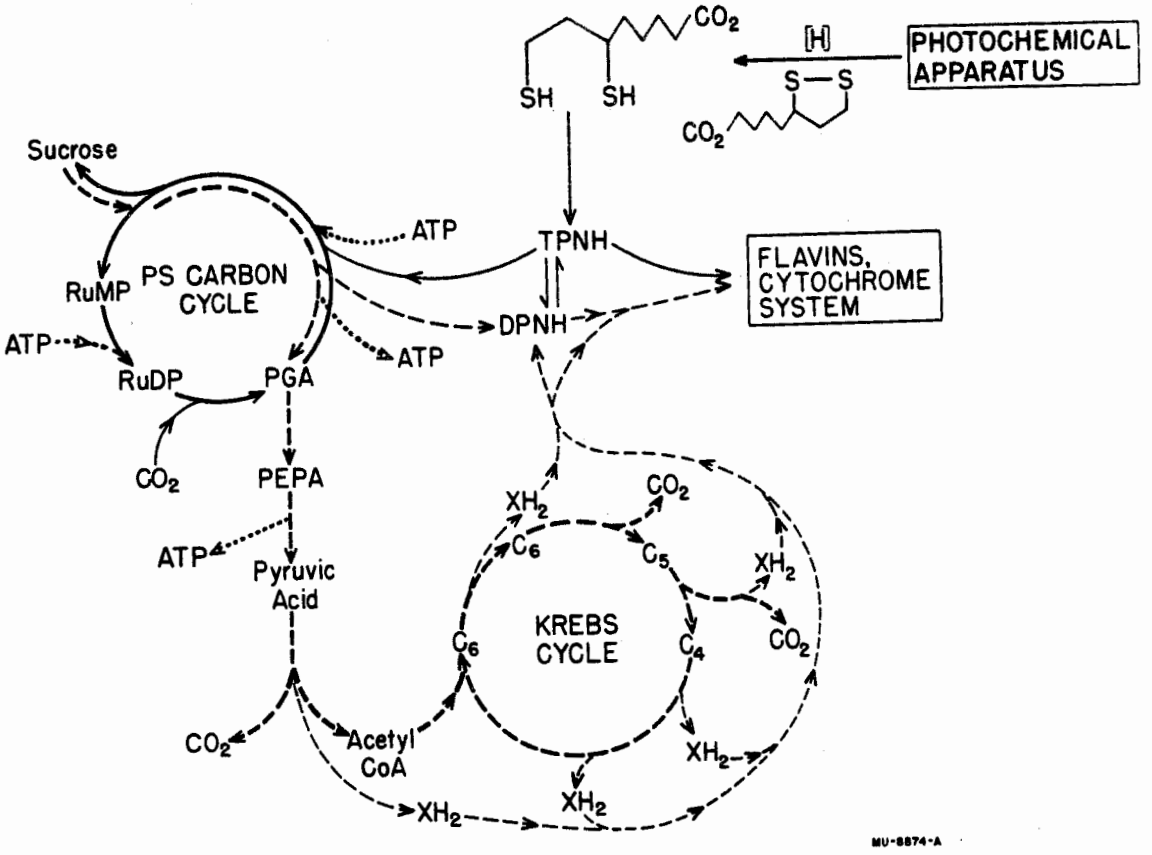

Fig. 29a Diagram of the Suggested Nature of the Photochemical Apparatus and Its Relationship to Other Functions

----- Oxidative, or Respiratory, Pathways

——— Reductive, or Photosynthetic, Pathways

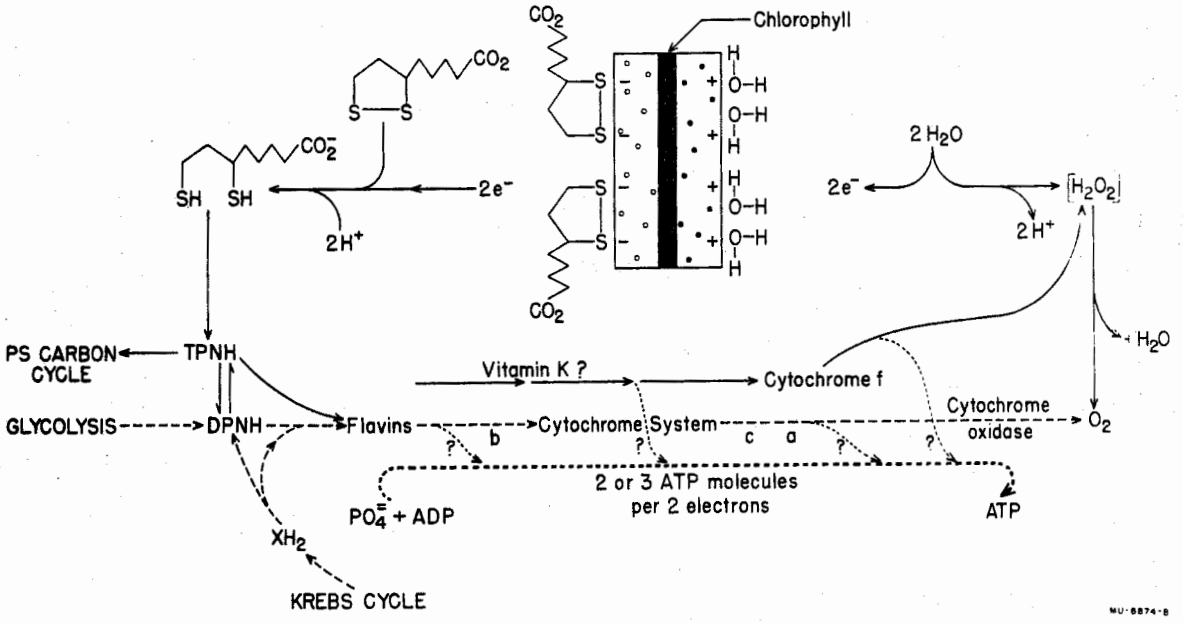

Fig. 29b Diagram of the Suggested Nature of the Photochemical Apparatus and Its Relationship to Other Functions

----- Oxidative, or Respiratory, Pathways

——— Reductive, or Photosynthetic, Pathways

the energy acceptor -- must be present in concentrations equal to or as great as that of the chlorophyll. There is no single molecular species present in that amount except water or its close relatives (such as, possibly, carotenoid alcohols). So here we are now putting the water molecule at one end of a photobattery, if you like (Fig. 29), with the disulfide at the other. The conducting electrons created in the chlorophyll layer migrate to one side of the double layer, and the holes are immediately trapped on the other side by electron donation from the water molecules, which are present in large amounts, so the back reaction cannot go on. We have, then, long-lived electrons, which can wait at the opposite side of the layer for the sulfur compound to go through its cycle several times and pick them all up. This is the suggestion as it now stands, and it is consistent with most of the information we have today.